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PROTEIN PHOSPHORYLATION CATALYZED BY CYCLIC AMP-DEPENDENT AND CYCLIC GMP-DEPENDENT PROTEIN KINASES

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INTRODUCTION

Phosphorylation-dephosphorylation has long been recognized as a process of reversible, covalent protein modification (1). These reactions are thought to serve a regulatory function in the short-term control of a variety of cellular processes. The state of phosphorylation of a given protein is controlled by the activity of the specific protein kinase or phosphoprotein phosphatase that catalyzes the respective incorporation or removal of phosphate. Protein kinases have been classified according to the specific effector molecules that directly interact with them and regulate their function (2). Thus, distinct classes of protein kinases are known to exist whose actions depend upon the second messengers adenosine 3':5'-monophosphate (cAMP) (3), guanosine 3':5'-monophosphate (cGMP) (4), or calcium ion (5–7), or on double-stranded RNA (8–10). In addition, there is a group of nonspecified protein kinases which are apparently independent of control via specific regulatory effectors. This review is restricted to the phosphoryla-

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tion of enzymes and other proteins by the cyclic nucleotide–dependent protein kinases.

Since the discovery of cAMP-dependent protein kinase (cAMP kinase) in rabbit skeletal muscle (3), this enzyme has been found to play a critical role in subserving the action of cAMP in a great number of processes in which this cyclic nucleotide is involved in mammalian organisms. A continually expanding literature has appeared concerning the cAMP kinase in the decade since its discovery, and the reader is referred to previous reviews of this enzyme in particular and protein kinases in general (2, 11–16). A distinct cGMP-dependent protein kinase (cGMP kinase), which is preferentially activated by cGMP rather than cAMP, was originally discovered in various species of arthropods (4, 17). The cGMP kinase was subsequently identified in mammalian tissues (18–21) and has been the topic of a recent review (22). The number of reviews of the cyclic nucleotide-dependent protein kinases indicates the degree to which advances have been made in the understanding of molecular details and the regulation of these enzymes. However, until recently the accumulation of information concerning the cGMP kinase had lagged behind that of the cAMP-dependent enzyme. This was accounted for, in part, by the more restricted tissue distribution and lower tissue concentration of the cGMP kinase, as well as the unavailability of a highly purified preparation of the enzyme. The cGMP kinase has recently been isolated in a homogeneous form from both bovine lung (23, 24) and heart (25), and progress in its study has advanced to the point that detailed comparisons have been made between the two cyclic nucleotide-dependent enzymes. The cGMP and cAMP kinases share several properties including some physical characteristics, similar substrate specificities, and the ability to undergo self-phosphorylation. However, the two kinases differ in several unique properties including subunit structure, mechanism of activation by cyclic nucleotides, and response to inhibitor and modulator proteins as well as in cyclic nucleotide specificity. The present chapter reviews recent work that allows comparisons of distinct similarities and differences between cGMP and cAMP kinases. Considerations of space have precluded a comprehensive review of the details of both enzymes; rather, comparisons of the two kinases are made that might be helpful in assessing how alike or different the proteins are both biochemically and, of most importance, functionally. We concentrate primarily on (*a*) the physical-chemical properties of the enzymes, (*b*) their responses to protein regulators, and (*c*) their protein substrate specificities. In this respect, consideration is given to the criteria (2, 26) that should be satisfied to establish whether or not a particular phosphorylation reaction catalyzed by either protein kinase is of physiological significance.

Although an involvement of the cAMP kinase in each of the physiological effects that cAMP is known to mediate has not been documented, the

role of the enzyme is considered central to the transduction of cAMP action (27). The biological role(s) of cGMP as a regulatory effector in the expression of hormone or neurohumor action remains less clear (28). It is known, however, that in many instances opposite physiological effects are associated with elevations in the intracellular levels of cAMP and cGMP (28, 29). It is not known whether all of the possible influences of cGMP are mediated through the activation of the cGMP kinase. If this is the case, one would expect different substrate proteins to be phosphorylated by the two cyclic nucleotide–dependent protein kinases. The occurrence of cGMP binding proteins without apparent phosphotransferase activity (30, 31) suggests that cGMP may also be associated with processes not involving the protein kinase. However, the relationship of the phosphorylation of specific substrate proteins by the cGMP kinase to changes in the function of those proteins and those of the entire cell should help to shed light on the role of this cyclic nucleotide.

PHYSICAL-CHEMICAL PROPERTIES

Subunit Structure and Mechanism of Activation

cAMP-DEPENDENT PROTEIN KINASE Two isozymes of the cAMP kinase have been identified, and the relative amounts of each form vary from tissue to tissue (32, 33). Numerous studies have indicated that both isozymes of the protein kinase are composed of two dissimilar subunits, termed regulatory and catalytic, respectively (reviewed in 11–16). The mechanism of activation has been shown to be as follows:

$$\underset{\text{(inactive holoenzyme)}}{R_2C_2} + 4\ cAMP \leftrightharpoons \underset{\text{(active catalytic subunit)}}{R_2 \cdot cAMP_4} + 2C.$$

Binding of cAMP to the regulatory subunit of the inactive holoenzyme results in the dissociation of the tetramer into a regulatory subunit dimer containing 4 mol bound cAMP (34, 35) and free catalytic subunits possessing phosphotransferase activity. The differences in the isozymes appear to be due to differences in the regulatory subunits (16, 33); the catalytic subunits appear to be identical in most properties examined (33). The physical and functional properties of the subunits of cAMP kinase have been extensively reviewed (15, 16, 36).

cGMP-DEPENDENT PROTEIN KINASE Early studies with partially purified preparations of cGMP kinase suggested that the mechanism of activation of the enzyme by cyclic nucleotide was analogous to that of the cAMP-dependent enzyme (19, 37–42). In the presence of high cGMP con-

centrations and histone (39), partially purified preparations of the cGMP kinase from fetal guinea pig lung (40), fetal calf heart (41), and bovine aorta (42) have been reported to be modified such that a putative catalytic subunit was formed. However, this apparent dissociation of the kinase did not occur in the presence of lower concentrations of cGMP that fully activated the enzyme (22), and a cGMP-binding regulatory subunit has not consistently been recovered (39, 42). It appears, then, that dissociation of the mammalian cGMP kinase may not be a physiological process (39). These considerations have been reviewed by Kuo et al (22).

The highly purified enzyme from silkworm pupae (43) or the homogeneous enzymes from bovine lung (23, 24, 44) or heart (25) are not dissociated into subunits by cGMP. Cyclic GMP-binding and protein kinase activities have, however, been separated after limited proteolysis of the enzyme (45). The enzyme purified from bovine lung is composed of only one type of subunit that apparently contains both cyclic nucleotide-binding and phosphotransferase activities on a single polypeptide chain (23, 24). Therefore, the mechanism of activation of the cGMP kinase is different from that of the cAMP kinase (23, 24, 44, 46) and can be depicted as follows:

$$\underset{\text{(inactive form)}}{E_2} + 2\ cGMP \leftrightharpoons \underset{\text{(active form)}}{E_2 \cdot cGMP_2}.$$

The molecular weight of the subunit, E, has been reported to be from 74,000 to 82,000 and that of the dimeric, native enzyme to be 150,000 to 160,000 (23–25, 44). Interchain disulfide bridges are probably involved in linking the two subunits together (44, 47). The physical and hydrodynamic properties of the holoenzyme, including Stokes radius, sedimentation coefficient, frictional coefficient, axial ratio, isoelectric point, and amino acid composition, have been determined (24, 44, 48).

Homology Theory

Although the subunit structure and mode of activation of the two protein kinases are different, a number of similarities exist between them. Both enzymes have a similar size and an asymmetric shape (35, 44, 48). Their kinetic properties, including K_m for ATP, metal ion requirements, and binding affinities for the respective cyclic nucleotides, are also alike (23, 24, 35, 44, 48). However, recent reevaluation of the stoichiometry of cAMP binding to the regulatory subunit of cAMP kinase has indicated that 2 mol rather 1 mol (15, 16, 33) of cAMP are bound per monomer at equilibrium (34, 35). The cGMP kinase binds only 1 mol of cGMP per monomer (24, 35, 44). In addition, the binding sites for cAMP on cAMP kinase are independent (33, 34, 49) while those for cGMP on the cGMP kinase exhibit

positively cooperative interactions (49, 50). Both enzymes catalyze their own self-phosphorylation (46, 51, 52) and have other similarities in substrate specificity (35, 36, 46, 48). The amino acid composition of cGMP kinase is similar to that of the Type II isozyme (holoenzyme) of cAMP kinase, suggesting a degree of sequence homology (48). It is interesting to note that there is only minimal cross-immunoreactivity between bovine cGMP kinase and antibodies to Type I regulatory subunit of cAMP kinase from the same species (53).

Based on this information about cGMP and cAMP kinases, Lincoln & Corbin (48) and Gill et al (44) have proposed that the two enzymes are homologous proteins. Sequence data required to confirm or refute this suggestion are not yet available. Possible molecular structures of the enzymes have been presented (36, 46). The cGMP kinase has been proposed to be composed of two identical subunits, each with cyclic nucleotide binding and catalytic domains, that are associated in an antiparallel fashion. The structure of cAMP kinase is suggested to have a similar dyad axis of symmetry, but there is a discontinuity in each chain such that activation by cAMP allows the dissociation of the holoenzyme into regulatory and catalytic subunits. These models suggest parallel evolution from an ancestral protein kinase (36, 48), and possible evolutionary schemes have been discussed (54).

PROTEIN REGULATORS OF KINASE ACTIVITY

Inhibitor Protein(s) of cAMP-Dependent Protein Kinase

The cAMP and cGMP kinases are regulated by heat-stable proteins that are inhibitory (55) and stimulatory (56) to their activities, respectively. The heat-stable inhibitor protein of the cAMP-dependent enzyme (55, 57) has recently been purified to homogeneity from skeletal muscle (58), brain (59), and heart (60). The protein is a potent inhibitor of the catalytic subunit of cAMP kinase and acts competitively with respect to protein substrates (58). The inhibitor has no effect on the activity of cGMP kinase (56, 59). A pertinent question yet to be answered is why the inhibitor is selective for the cAMP kinase, especially if it is substrate-like, insofar as the cAMP kinase is concerned, and the two cyclic nucleotide–dependent kinases have similar substrate specificities. The physiological role of the protein kinase inhibitor is not entirely clear, but it is thought to prevent the action of any free catalytic subunit present under basal levels of cAMP (61). Recently, two other proteins inhibitory to cAMP kinase have been reported, one from rat testis that is also inhibitory to cAMP phosphodiesterase (62) and another from brain that inhibits the action of several classes of protein kinase, including the cGMP kinase (63).

Stimulatory Modulator of cGMP-Dependent Protein Kinase

Kuo and associates (39, 56, 64, 65) have studied a heat-stable protein that stimulates the activity of the cGMP kinase under certain conditions in vitro. This topic has been reviewed recently (22), but certain points of comparison with the inhibitor protein of cAMP kinase should be considered. Unlike the inhibitor of cAMP kinase which interacts directly with the catalytic subunit of the enzyme (58), the modulator protein of the cGMP kinase exerts its action by interacting with substrate proteins thus making them more effective substrates (65). This effect of the modulator protein was seen predominantly with histones as substrates (65, 66) although the phosphorylation of ribosomal proteins was also enhanced by its presence (67). The modulator did not augment the phosphorylation of several other nonhistone proteins or peptides (65). The protein affects only the rate of substrate phosphorylation and not the specific sites modified (65, 66). Its physiological relevance remains to be determined. High magnesium concentrations have been reported to augment the phosphorylation of histones by the cGMP kinase (21, 25, 43, 68), and this effect is also probably substrate-directed (69). Phosphorylation of various histone fractions by cGMP kinase is enhanced by polydeoxyribonucleotides, leading Hashimoto et al to suggest that nucleosome core histones may be preferential substrates for the enzyme (70). Changes in the tissue levels of the protein kinase inhibitor and stimulatory proteins will not be discussed.

SUBSTRATE SPECIFICITY

Phosphorylation of Purified Substrate Proteins

Many, albeit not all, of the cAMP kinase–catalyzed protein phosphorylation reactions came to the attention of investigators for examination in vitro because of known physiological functions of cAMP. By contrast, most of the cGMP kinase–catalyzed reactions have been discovered as a result of studies to determine whether known substrates of the cAMP kinase would also be effective substrates for cGMP kinase. As will be seen in this section, which will emphasize findings relative to the cGMP kinase rather than exhaustively reviewing the more extensive background work involving the cAMP kinase, the two enzymes appear to have overlapping substrate specificities although certain differences are apparent.

HISTONES Studies with partially purified preparations of cGMP kinase indicated that histones were better phosphate acceptors than were nonhistone proteins such as casein and protamine (41, 68, 71). The latter proteins are known to serve as substrates for cAMP kinase (3, 11, 16). The sub-

fractions of histone most readily phosphorylated by the cGMP kinase are H2B > H1 > H2A, with the arginine-rich subfractions H3 and H4 being much poorer substrates (25, 66, 70, 72). The H2B subfraction is the best histone substrate for the cAMP kinase (70, 72, 73), although the enzyme catalyzes the phosphorylation of all five fractions (66). Early studies suggested that the two protein kinases were distinguishable in their substrate specificities toward histones. Takai et al (68) and Nishiyama et al (74) reported different patterns of phosphopeptides in tryptic peptide maps of mixed histone that had been phosphorylated by either cGMP or cAMP kinases from silkworm pupae or bovine cerebellum.

Numerous investigators have determined the sites phosphorylated in histones H1 (75–77), H2B (77–80), and H2A (77) by the cAMP kinase. Hashimoto et al (72, 78) and Kuroda et al (80) showed that the same residues in histones H1 and H2B are phosphorylated by both of the protein kinases. These studies did not examine the kinetics of the phosphorylations catalyzed by the two enzymes. However, the relative ratio of phosphate associated with serine-32 to that associated with serine-36 in histone H2B was much higher with cGMP kinase than with cAMP kinase (66, 72). Subsequently, Yamamoto et al (66) demonstrated that apparently identical sites in histone H4 are phosphorylated by both enzymes. The only phosphorylation of histones by cyclic nucleotide-dependent protein kinases that is known to occur in vivo is that of site-37 in histone H1 by the cAMP kinase (75, 76, 81).

PHOSPHORYLASE KINASE Phosphorylase *b* kinase, a natural substrate for cAMP kinase (82), was reported not to be phosphorylated or to undergo an activity change when incubated with the cGMP kinase (45, 68, 71, 74). However, the availability of homogeneous preparations of cGMP kinase has allowed a reexamination of the substrate specificity of the enzyme and, contrary to initial reports, the purified cGMP kinase has been shown to phosphorylate (48, 54) and activate (83, 84) phosphorylase kinase from rabbit skeletal muscle. Approximately 20 times as much cGMP as cAMP kinase was required to affect the same degree of change in either of these processes. It is not known whether cGMP kinase phosphorylates the same sites on the α- and β-subunits of phosphorylase kinase as does the cAMP kinase (73, 85). The phosphorylation of phosphorylase kinase by cGMP kinase has not been demonstrated in vivo, and the physiological significance of the in vitro observation remains unknown. The levels of cGMP in skeletal muscle are known to be under neurohumoral regulation (86). However, the low level of cGMP kinase in skeletal muscle (30) and the lower efficiency of this enzyme in catalyzing the phosphorylation of phosphorylase kinase

suggest that an activation of phosphorylase kinase in vivo by cGMP kinase would be unlikely.

GLYCOGEN SYNTHASE The cAMP kinase phosphorylates and inactivates glycogen synthase (82). An early report indicated that cGMP kinase had no such effect on this enzyme (74). However, Lincoln & Corbin (48, 54) recently demonstrated the phosphorylation of skeletal muscle glycogen synthase by the cGMP kinase. The rate and extent of phosphorylation of the enzyme was less with cGMP kinase than with cAMP kinase, although phosphorylation by cGMP kinase was associated with a conversion of glycogen synthase from the I- to the D-form (48). The amino acid sequence around one of the sites in glycogen synthase phosphorylated by the cAMP kinase has been determined (87, 88). The particular site(s) modified by the cGMP kinase remains unknown, but both protein kinases phosphorylated a portion of the enzyme that is sensitive to proteolysis by trypsin (48).

HORMONE-SENSITIVE LIPASE A partially purified preparation of hormone-sensitive lipase from chicken adipose tissue was activated to the same extent by cGMP kinase as by cAMP kinase (83); however, more of the former enzyme was required to accomplish this activation. The lipase from both rat (89, 90) and human (91) adipose tissue is also activated by cAMP kinase and, to a relatively minor degree, by cGMP kinase (83). The degree of phosphorylation of the lipase by the cGMP kinase is not known, but the activation by the kinase was reversed by purified phosphoprotein phosphatase (83). Activation of the lipase by a cGMP-dependent phosphorylation in vivo has not been reported. However, if this type of regulation were to occur, the effect would apparently be lipolytic, as is produced by increased levels of cAMP.

PYRUVATE KINASE There is good evidence that pyruvate kinase is regulated by phosphorylation by the cAMP kinase (reviewed in 92). Only the L-type isozyme of the enzyme is phosphorylated (93). Hepatic pyruvate kinase is phosphorylated by the cAMP kinase both in vitro (94, 95) and in vivo (96, 97). The sites of phosphorylation in both the rat (98) and pig (99) enzymes have been determined. The decrease in pyruvate kinase activity caused by its phosphorylation is the consequence of lowered affinity of the enzyme for the substrate phosphoenolpyruvate (100, 101). Lincoln & Corbin (48, 54) have demonstrated the phosphorylation of purified pyruvate kinase by the cGMP kinase. This incorporation of phosphate was claimed to be associated with an inhibition of the activity of pyruvate kinase (48). Detailed kinetic constants were not obtained, but the rate of phosphorylation of pyruvate kinase by cGMP kinase was only approximately 5% of that

catalyzed by the catalytic subunit of cAMP kinase. The physiological significance, if any, of this in vitro phosphorylation remains to be established.

FRUCTOSE-1,6-BIPHOSPHATASE Both protein kinases have been reported to phosphorylate fructose-1,6-biphosphatase from rat liver (48, 54, 102). Phosphorylation of this substrate by cAMP kinase proceeded at a much slower rate than did the phosphorylation of phosphorylase kinase (54, 103). Interestingly, Riquelme et al (103) reported that under the same conditions cAMP kinase did not phosphorylate fructose-1,6-biphosphatase from mouse liver or pig kidney. The enzyme from rat liver is phosphorylated in vivo, probably by the cAMP kinase, and this phosphorylation is associated with an increase in enzyme activity (102). The functional influence of the phosphorylation by cGMP kinase has not been reported. Again, with fructose-1,6-biphosphatase as substrate, cAMP kinase was reported to be a more effective catalyst than was the cGMP-dependent enzyme (48, 54).

TROPONIN Two groups of workers (104, 105) found that troponin from cardiac muscle serves as a substrate for cGMP kinase, as was previously recognized to be the case for cAMP kinase (106, 107). Only the inhibitory subunit of troponin (TN-I) was stoichiometrically phosphorylated (104, 105) by either enzyme, and similar results were obtained with either troponin itself or with troponin-tropomyosin complex (104). The major site in TN-I phosphorylated by cAMP kinase has been determined (108, 109), but the site(s) phosphorylated by the cGMP kinase has not been sequenced. The cGMP and cAMP kinases did not catalyze additive phosphorylation of TN-I, suggesting that the same site was phosphorylated by both enzymes (104, 105). The kinetic constants reported by Blumenthal et al (104) showed that cardiac troponin was a better substrate for the cAMP kinase than for the cGMP-dependent enzyme. However, as pointed out by Lincoln & Corbin (54, 105), the rate of phosphorylation of TN-I by cGMP kinase was relatively high compared to that catalyzed by cAMP kinase, and TN-I may contain additional specificity determinants for the cGMP kinase that other protein substrates lack. A β-adrenergic-induced elevation in the cardiac content of cAMP is associated with increased contractility (110) and has been correlated with an enhanced phosphorylation of TN-I in vivo (111, 112). On the other hand, an increase in cardiac cGMP content produced by cholinergic stimulation is associated with a depression in contractility (113) and has been correlated with a dephosphorylation of TN-I (111). The inability to observe an in vivo phosphorylation of cardiac TN-I by the cGMP kinase (112) is probably explained by the fact that the V_{max} for the phosphorylation of this protein and the tissue concentration of the cGMP

kinase are both low compared to the corresponding parameters for the cAMP kinase (30, 104).

CHOLESTEROL ESTER HYDROLASE Cholesterol ester hydrolase from chicken adipose tissue (114) and rat adrenal gland (115) has been shown to be activated by the cAMP kinase. In adipose tissue, the hydrolase activity may be identical with the hormone-sensitive lipase (114). Khoo et al (83) showed that cGMP kinase also caused the activation of the hydrolase from chicken adipose tissue and, to a limited extent, that from the rat adrenal gland. Because of problems in obtaining a purified preparation of cholesterol ester hydrolase, the stoichiometry and kinetics of phosphorylation of the enzyme that is associated with its activation by either protein kinase remains unknown. The physiological significance of a possible cGMP-mediated phosphorylation and activation of cholesterol ester hydrolase remains obscure. However, such a role of the cGMP kinase would be consistent with recent reports (116, 117) of the involvement of cGMP in mediating steroidogenesis in the adrenal gland, although this proposal has been contested (118).

OTHER PROTEINS Both protein kinases from silkworm pupae have been reported to phosphorylate, in vitro, similar ribosomal proteins from rat liver (67). Myelin basic protein is also a substrate for both cGMP kinase (22) and cAMP kinase (119, 120), but a direct comparison of the kinetics of phosphorylation is lacking.

Several proteins that are either physiologically significant substrates or useful model substrates for cAMP kinase have not yet been evaluated as substrates for the cGMP kinase. These include denatured chicken lysozyme (121), protein phosphatase inhibitor-1 from rabbit skeletal muscle (122), phosphofructokinase from rabbit skeletal muscle (103) and pig kidney (123), RNA polymerase II from calf thymus (124; reviewed in 2), and, as has recently been reported, the myosin light chain kinase from avian smooth muscle (125). In addition, various aromatic amino acid hydroxylating enzymes can be phosphorylated in a cAMP-dependent manner that results in an alteration in the kinetic properties of these enzymes. Tyrosine hydroxylase (126) from various tissues has been the most extensively studied of these enzymes (reviewed in 2), but phenylalanine hydroxylase (127, 128) and tryptophan hydroxylase (129) may also fall into this category.

Native glycogen phosphorylase is not a substrate for cAMP kinase (121, 130) even though the denatured protein (121), as well as a synthetic peptide corresponding to the site phosphorylated by phosphorylase kinase (130), will serve as phosphate acceptors. Glycogen phosphorylase is also not a substrate for the cGMP kinase (D. B. Glass, E. G. Krebs, unpublished).

Autophosphorylation of Protein Kinases

cAMP-DEPENDENT PROTEIN KINASE Erlichman et al (51) first reported that cAMP kinase from bovine cardiac muscle catalyzed the stoichiometric phosphorylation of its own regulatory subunit. This property is unique to the Type II isozyme of the enzyme from various tissues and species (131–133) and can involve either soluble or membrane-bound forms (134). Autophosphorylation of the Type I kinase apparently does not occur (33). An exception to this is the regulatory subunit of the Type I protein kinase from an S49 mouse lymphoma cell line which has been observed to be predominantly in a phosphorylated form in intact cells (135); however, the kinase catalyzing this phosphorylation has not been clearly demonstrated. The mechanism by which the catalytic subunit of the kinase incorporates phosphate into the regulatory subunit of the enzyme is an intramolecular reaction occurring within the undissociated holoenzyme (136, 137). Heat-denatured regulatory subunit (51) or regulatory subunit in the presence of excess cAMP (88) will serve as substrate for catalytic amounts of the catalytic subunit. Under the appropriate conditions, this reaction is reversible (138). The occurrence of a phosphoprotein phosphatase that acts on the free phosphorylated form of Type II regulatory subunit, but poorly on the phosphorylated holoenzyme, has been reported (139). The sequence of amino acids around the phosphorylated seryl residue has been determined (88). Treatment of the Type II regulatory subunit with either trypsin or 2,3-butanedione reduced its ability to serve as a substrate for the phosphorylation reaction (34, 35). If the phosphorylated form of Type II regulatory subunit was treated with trypsin, the protein-bound phosphate appeared in a 39,000 dalton proteolytic fragment (34, 35).

The phosphorylation of the Type II protein kinase alters its sensitivity toward cAMP (51) such that dissociation of the holoenzyme is favored (140–142). The phospho-form of the holoenzyme has a lower dissociation constant for cAMP than does the dephospho-form of the enzyme (33). Also, the phosphorylated Type II regulatory subunit has a reduced rate of reassociation with free catalytic subunit in the absence (140) or presence (142) of cAMP. There have been few studies addressing the important question of the phosphorylation state of the protein kinase in vivo and its possible hormonal regulation. Recent work by Rangel-Aldao et al (143) suggests that the regulatory subunit of protein kinase in bovine cardiac tissue is predominantly in the phosphorylated form. If this is generally the case, then the possible regulation of the phosphoprotein phosphatase catalyzing the dephosphorylation reaction gains added importance in understanding the biological significance of the autophosphorylation phenomenon.

cGMP-DEPENDENT PROTEIN KINASE DeJonge & Rosen (52) initially reported that the purified cGMP kinase from bovine lung was also a substrate for its own phosphotranferase activity. This intramolecular reaction proceeds to the extent of 2 mol of phosphate per mol of holoenzyme (46), but the nature of the sites phosphorylated has not been determined. Arginine residues appear to play a role in the autophosphorylation because modification of the enzyme with 2,3-butanedione inhibits the phosphorylation reaction (46). In these same studies, mild treatment of the cGMP kinase with trypsin reduced the autophosphorylation of the enzyme without greatly altering the phosphotransferase activity toward histone H2B as substrate. The functional consequences of the autophosphorylation remains unclear at this time, but it is not an intermediate step in the phosphotransferase reaction (52). Cyclic GMP itself has been reported to inhibit the autophosphorylation reaction, while cAMP and histone enhance it (46, 52).

Phosphorylation of Endogenous Substrate Proteins

PHOSPHORYLATION CONTROLLED BY cAMP A number of known protein substrates of the cAMP kinase whose phosphorylation is thought to be of physiological significance have, indeed, been shown to be phosphorylated in vivo under appropriate conditions of hormonal stimulation. Many of the proteins previously discussed fall into this category, and the reader is referred to recent reviews (2, 16) of this topic. These in vivo substrates of the cAMP kinase include phosphorylase *b* kinase (144), pyruvate kinase (96), fructose-1,6-biphosphatase (102), cardiac troponin (111, 112), and histone H1 (75, 76).

Other endogenous substrates for the cAMP kinase are less well characterized. In most cases, phosphorylation of these endogenous proteins has not been clearly demonstrated in vivo, but, rather, cAMP-stimulated phosphorylation has been observed in cell-free tissue preparations. No further comment will be made on a number of these endogenous substrates for the cAMP kinase, and the reader is directed to a review covering this area (15). However, notable progress has been made in characterizing certain endogenous substrates of cAMP kinase, and these are discussed below.

Ueda et al (145) detected a cAMP-dependent phosphorylation of several proteins in synaptic membrane–enriched preparations from rat brain. Further work resolved one of these proteins into two phosphoproteins, termed Ia (86,000 daltons) and Ib (80,000 daltons) (146). These endogenous substrates are unique to neural tissue, have been purified to homogeneity, and have been characterized in terms of their physicochemical properties (146). The ontogeny of these proteins during synaptogenesis has been described (147), but their function in the synapse remains speculative. Proteins Ia and Ib are also phosphorylated in a calcium-dependent manner during depolari-

zation of cerebral cortical slices (148, 149), but this process involves different sites of phosphorylation from those produced by cAMP (150). The other endogenous substrate described by Ueda et al (145), termed phosphoprotein II, has a widespread occurrence in mammalian tissues (151), and the extent of its phosphorylation is apparently controlled by steroid hormones (152). This protein has tentatively been identified as the regulatory subunit of Type II cAMP kinase (146, 153) (see section on autophosphorylation of protein kinases).

Pastan and co-workers reported that the actin binding protein, filamin, from smooth muscle (154), fibroblasts, and other nonmuscle cells (155) is phosphorylated in response to cAMP. Purified cGMP kinase was not tested with filamin as substrate, but the phosphorylation of filamin in particulate fractions of vas deferens was not affected by cGMP (154).

Maller & Krebs demonstrated that microinjection of purified catalytic subunit of mammalian cAMP kinase into intact *Xenopus* oocytes inhibited progesterone-induced oocyte maturation (156). It was concluded that catalytic subunit was able to maintain an endogenous oocyte protein in a phosphorylated state, and that this was sufficient to prevent the meiotic maturation usually induced by progesterone. Interestingly, Maller et al (157) have reported that microinjection of homogenous cGMP kinase into *Xenopus* oocytes also inhibits progesterone-induced meiotic cell division. The regulatory subunit of cAMP kinase and the heat stable inhibitor protein (55) were able to directly induce meiotic division in the absence of progesterone (156). These results suggested that the catalytic subunit of *Xenopus* cAMP kinase could be inhibited in intact oocytes. An endogenous protein kinase present in oocytes phosphorylated a synthetic substrate when it was injected into these cells. Microinjection into intact oocytes of a synthetic peptide known to be a substrate for the cAMP kinase (158) resulted in an in vivo phosphorylation of the peptide, indicating that intracellularly it was accessible to the protein kinase (157). The characteristics of the putative endogenous protein substrate for protein kinase have not been investigated.

PHOSPHORYLATION CONTROLLED BY cGMP Little evidence is available to suggest that any of the proteins that serve as substrates for cGMP kinase in vitro are also phosphorylated in a physiologically significant manner in vivo. However, recent reports suggest that specific physiological substrate proteins do exist for the cGMP kinase (159–165). A number of proteins endogenous to various cell-free tissue preparations are phosphorylated in response to cGMP, but the actual in vivo phosphorylation of any of these proteins by cGMP kinase has not been demonstrated. As opposed to many of the substrates for cAMP kinase, whose identity and function are known, little is known concerning the characteristics of specific endogenous substrates for the cGMP kinase.

Casnellie & Greengard (159) first demonstrated that the phosphorylation of two membrane proteins in mammalian smooth muscle was stimulated preferentially by cGMP. The molecular weights of these two polypeptide chains were 130,000 and 100,000, and seryl residues were the predominant phosphorylated sites. Similar results were obtained by Wallach et al (154) in guinea pig vas deferens, although in addition to the 130,000 dalton protein, they reported an endogenous substrate of 240,000 molecular weight, which was also membrane associated. The cGMP-stimulated phosphorylation of a specific 86,000 molecular weight protein of membranes from the brush boarder of rat intestinal epithelium has been reported by deJonge (160). The phosphorylation of a different protein was selectively stimulated by cAMP in the cytosolic fraction from the same tissue. Shlatz et al (161) also observed that cGMP was more effective than cAMP in enhancing phosphate incorporation into purified microvillus membranes prepared from intestinal epithelium.

Recently, evidence for the existence of endogenous substrates for cGMP kinase in neural tissue has been reported (162–164). Phosphorylation of a low molecular weight polypeptide (23,000 daltons) was produced by cGMP in a soluble fraction from rabbit cerebellum (162). This protein appeared to be phosphorylated only by cGMP kinase, and not by cAMP kinase (either Type I or Type II), and had a restricted distribution in the central nervous system (162). With this report of a specific substrate of the cGMP kinase in mammalian cerebellum, all the components of the cGMP effector system, including cGMP itself, guanylate cyclase, and the protein kinase have been reported to occur in high amounts in this tissue (29). Boehme et al (163) recently reported that cGMP, but not cAMP, enhanced the phosphorylation of several proteins, including a major polypeptide of 60,000 molecular weight, in synaptosomal membranes prepared from several areas of human brain obtained postmortem. Proteins of a similar molecular weight (68,000 and 62,000) were shown to be phosphorylated in a membrane preparation from nerve roots of a molluscan species after exposure to cGMP but not cAMP (164). A brief report indicated that addition of cGMP or carbachol to cultured horse peripheral lymphocytes doubles the degree of phosphorylation of total nuclear acidic proteins (165).

Determinants of Substrate Specificity

cAMP-DEPENDENT PROTEIN KINASE Because early studies revealed no apparent similarities between amino acid sequences of phosphorylation sites in substrates of cAMP kinase, it was initially assumed that a particular tertiary structure of substrates was required for their phosphorylation (12, 85). However, it was found that denaturation of certain proteins greatly

increased their ability to be phosphorylated by the kinase (121, 166). Also, Daile & Carnegie reported that peptides isolated from proteolytic digests of myelin basic protein served as substrates for cAMP kinase (167). These studies demonstrated that phosphorylation of proteins was not dependent on their native structure and indicated that their primary structure was important in determining specificity. The importance of basic amino acid residues in dictating specificity was first suggested by studies of the phosphorylation of genetic variants of β-casein (168) and as a result of studies on peptides derived from histone (77). Studies with synthetic peptide substrates corresponding to sequences around phosphorylation sites in myelin basic protein (169), chicken lysozyme (170), pyruvate kinase (158, 171), histones (69, 172, 173), and the inhibitory subunit of cardiac troponin (174) have all confirmed the importance of basic residues, especially arginines, as determinants of specificity of the cAMP kinase. The sequence of amino acids around the sites phosphorylated in native pyruvate kinase from rat (98) and pig (99) liver suggested that two adjacent arginine residues were critical. This concept was shown to be the case by studies with synthetic peptides corresponding to these sites (158, 171). In addition arginine, arginyl-arginine, and polyarginine are all competitive inhibitors of the kinase (58, 175).

An examination of the extended sequences around phosphorylation sites in natural substrate proteins indicated that a number of these sites contain multiple basic residues in the proximity of, but amino-terminal to, the phosphorylated site (73, 88, 98, 99, 109, 122, 176). The type and exact position of the basic residues vary among substrates, however. Two general classes of primary sequences around phosphorylation sites in physiologically significant substrates have been noted. These are Arg-Arg-X-Ser(P) which is seen in the α-subunit of phosphorylase kinase (73), pyruvate kinase (98, 99), and the regulatory subunit of Type II cAMP kinase (88), and Lys-Arg-X-X-Ser(P) which occurs in the β-subunit of phosphorylase kinase (73) and in one site in glycogen synthase (88). In studies of the phosphorylation of herring protamines, Shenolikar & Cohen (177) addressed the question of the optimal distance of adjacent arginine residues from the phosphorylatable serine and concluded that with these substrates the presence of two amino acid residues between the diarginine and the serine was optimal. This is in contrast to studies using synthetic peptides of the general sequence $(Gly)_x$-Arg-Arg-$(Gly)_y$-Ala-Ser-Leu-Gly, where $x + y = 6$ (J. R. Feramisco, D. B. Glass, E. G. Krebs, submitted). The peptide $(Gly)_6$-Arg-Arg-Ala-Ser-Leu-Gly (corresponding to the general sequence Arg-Arg-X-Ser) was the optimal substrate. Peptides in which the arginine residues were in any other position relative to the serine exhibited marked increases in K_m or decreases in V_{max}, or both.

Serine residues in substrates appear to be much better phosphate acceptors than are threonines (158, 171). However, a threonine residue in a unique sequence is the site of phosphorylation of phosphoprotein phosphatase inhibitor-1 (122). Interestingly, Feramisco et al have shown that the cAMP kinase will phosphorylate a hydroxyproline residue in a synthetic peptide containing the other required specificity determinants; however, this peptide is an inferior substrate to the corresponding serine-containing peptide (178).

The specificity determinants of cAMP kinase have been compared to those of phosphorylase kinase using model synthetic peptide substrates, and the two have been found to be quite distinct (130, 158, 179, 180). These studies and the work of Kemp (174) have shown that the presence of a basic residue carboxy-terminal to the phosphorylated serine in substrates has a deleterious effect on their phosphorylation by the cAMP kinase. Synthetic peptides have proved useful as inhibitors of the protein kinase (170, 175) and in studies of the kinetic mechanism of the reaction catalyzed by the enzyme (172, 175; J. R. Feramisco, E. G. Krebs, in preparation), and should prove useful as probes of the active site of the enzyme.

The fact that not all synthetic peptides exhibit kinetic constants as favorable as those observed for the intact proteins from which they are modeled (170, 174) suggests that other factors, in addition to primary structure, influence the substrate specificity of the enzyme. It is probable that higher orders of structure, either contained in protein substrates or assumed by peptides at the active site, are of importance. It has recently been predicted by Small et al (181) and others (179, 182) that phosphorylation site sequences might be contained in a hydrogen-bonded β-bend structure at the surface of substrate proteins.

cGMP-DEPENDENT PROTEIN KINASE Less is known concerning the details of specificity determinants for the cGMP kinase. Because no information is available concerning the sequence of sites phosphorylated by the cGMP kinase in specific endogenous substrates for the enzyme, the determinants of substrate specificity have been investigated by the use of synthetic peptides. Initial studies showed that the pyruvate kinase sequence peptide (158) was phosphorylated by the cGMP kinase (48, 63), indicating that the enzyme is able to recognize a simple sequence of amino acids. In this particular sequence, arginines were required for adequate rates of phosphorylation. However, kinetic constants determined for both cyclic nucleotide–dependent protein kinases showed that this peptide is a relatively poor substrate for cGMP kinase compared to the cAMP kinase (48, 69, 183). A sequence from the α-subunit of phosphorylase kinase was also phos-

phorylated by cGMP kinase with similar kinetic constants to those obtained with the pyruvate kinase peptide (183).

In several peptide sequences, threonine did not substitute well for serine (48), and a carboxy-terminal basic amino acid separated from the serine by one residue was a negative determinant for the cGMP kinase (183; D. B. Glass, E. G. Krebs, unpublished). In these respects and in the optimal spacial arrangement of basic residues on the aminoterminal side of the phosphorylation site, the two protein kinases were alike (D. B. Glass, J. R. Feramisco, in preparation). However, the two enzymes are able to recognize different specificity determinants in the primary sequence of substrates (69). Synthetic peptides corresponding to the sequences around serine-32 and serine-36 in histone H2B were phosphorylated with different kinetic preferences by cGMP and cAMP kinases. These results were consistent with observations made concerning phosphorylation sites in intact histone H2B (66, 72). Kinetic studies with analogues of the serine-32–containing peptide, Arg-Lys-Arg-Ser-Arg-Lys-Glu, showed that this sequence was favorably phosphorylated by the cGMP kinase relative to the cAMP kinase predominantly because of the presence of the arginine-33 immediately carboxy-terminal to the serine residue (D. B. Glass, E. G. Krebs, in preparation). The only characterized, pure substrates known to be better substrates for cGMP kinase than for cAMP kinase are Arg-Lys-Arg-Ser-Arg-Lys-Glu and its analogues (69), a carboxy-terminal chymotryptic fragment from histone H1 (C. E. Zeilig, T. A. Langan, D. B. Glass, in preparation) and the free regulatory subunit of Type I cAMP kinase (184).

CONCLUSION

Although similar phosphorylations are catalyzed by the cGMP and cAMP protein kinases, distinct differences in their substrate specificities have been demonstrated. However, in studying and comparing the substrate specificities of the two cyclic nucleotide–dependent protein kinases, major challenges still face investigators. These include (*a*) determination of sequences around sites phosphorylated by the enzymes in endogenous substrates, especially those of the cGMP kinase, (*b*) correlation of kinetic parameters with the structural conformation of substrates, and (*c*) continued work on the molecular structures of the kinases themselves, particularly the active sites. In addition, selective inhibitors of each enzyme for use in vivo would be most helpful. As information is accumulated to satisfy the criteria required of a physiologically significant phosphorylation catalyzed by the cAMP or cGMP kinase, a more detailed understanding of the roles and actions of the cyclic nucleotides should become apparent.

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