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Mapping of the Adenosine 5′-Triphosphate Binding Site of Type II Calmodulin-Dependent Protein Kinase†

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ABSTRACT: The specificity of the ATP-binding site of the type II calmodulin-dependent protein kinase was probed with 25 analogues of ATP modified at various positions of the molecule. The analogues were compared by their ability to compete with ATP in the protein kinase reaction. The result of this comparison indicates that the enzyme is most sensitive to modifications at, or replacement of, the purine moiety. Changes at the triphosphate chain are much better tolerated, although the enzyme exhibited a selective sensitivity to changes in the conformation of this group. The smallest contribution to the specificity of ATP binding appears to be made by the ribose ring. The K_i values obtained for a subset of these analogues were compared to those previously reported for phosphorylase *b* kinase and the cyclic nucleotide dependent protein kinases [Flockhart, D. A., Freist, W., Hoppe, J., Lincoln, T. M., & Corbin, J. D. (1984) *Eur. J. Biochem. 140*, 289–295]. A striking similarity in the responses of these protein kinases to modifications of the ATP molecule suggests that the type II calmodulin-dependent protein kinase is related to these enzymes. Support for this conclusion was provided, recently, through comparisons of the deduced primary structures of the α and β subunits of the type II calmodulin-dependent protein kinase with the protein sequences of the catalytic subunits of phosphorylase *b* kinase and cAMP-dependent protein kinase [Hanley, R. M., Means, A. R., Ono, T., Kemp, B. E., Burgin, K. E., Waxham, N., & Kelly, P. T. (1987) *Science (Washington, D.C.) 237*, 293–297; Bennett, M. K., & Kennedy, M. B. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 1794–1798], which indicated areas of extensive homology.

Type II calmodulin-dependent protein kinase (CaM kinase II)[1] is a multifunctional protein kinase that has been isolated from rabbit liver (Ahmad et al., 1982; Payne et al., 1983), rabbit skeletal muscle (Woodgett et al., 1983), rat brain (Bennett et al., 1983; Kennedy et al., 1983), and several other tissues [reviewed by Nairn et al. (1985)]. The enzyme is most prominent in the brain (Bennett et al., 1983; Nairn et al., 1985) where it is thought to be involved in regulating synaptic activity (Browning et al., 1985; Nairn et al., 1985) and in prolonging the effects triggered by the transient Ca^{2+} signal (Miller & Kennedy, 1986; Lai et al., 1986; Schworer et al., 1986).

The enzyme isolated from rat brain has a native molecular weight of approximately 630 000 and is composed of α (M_r

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[1] Abbreviations: CaM kinase II, type II calmodulin-dependent protein kinase; EDTA, ethylenediaminetetraacetate; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; ApCpp, adenosine 5′-(α,β-methylenetriphosphate); AppCp, adenosine 5′-(β,γ-methylenetriphosphate); AppNp, adenosine 5′-(β,γ-imidotriphosphate); 2-Cl-ATP, 2-chloroadenosine 5′-triphosphate; etheno-ATP, 1,N^6-ethenoadenosine 5′-triphosphate.

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50000) and β (M_r 58000/60000) subunits [reviewed by Nairn et al. (1985)]; the subunit composition of the enzyme varies with different regions of the brain (McGuinness et al., 1985; Miller & Kennedy, 1985). Both subunits can be autophosphorylated, bind calmodulin in a Ca^{2+}-dependent manner, and appear to be structurally and functionally related (Hanley et al., 1987; Nairn et al., 1985).

The relationship between CaM kinase II and other protein kinases was investigated in this study by mapping of the ATP-binding site with 25 analogues of ATP and comparing the structural determinants for the interactions of ATP with this protein kinase and other protein kinases. The nucleotide specificity of CaM kinase II differed from that observed for the nuclear, cyclic nucleotide independent protein kinase (Baydoun et al., 1981) but proved to be similar to those described for phosphorylase *b* kinase and the cyclic nucleotide dependent protein kinases (Flockhart et al., 1984), which suggests that CaM kinase II may be related to the latter group of enzymes. Support for this was only recently provided when the deduced primary structures of the α and β subunits of CaM kinase II showed areas of extensive homology to the protein sequences of myosin light chain kinase, the γ subunit of phosphorylase *b* kinase, and the catalytic subunit of cAMP-dependent protein kinase (Hanley et al., 1987; Bennett & Kennedy, 1987).

A comparison of the ATP specificity of CaM kinase II with that of other kinases constituted the major objective of this study. In addition, this survey of ATP analogues was carried out in order to identify nucleotides with high affinity for the enzyme to be suitable for use in equilibrium binding studies and to provide an indication of which portions of the ATP molecule can be modified to yield potentially useful fluorescent probes or affinity and photoaffinity labels for future studies directed at the ATP-binding site of this calmodulin-dependent protein kinase.

Materials and Methods

Materials. [γ-^{32}P]ATP was purchased from ICN Biomedicals, Inc. The synthetic peptide substrate Pro-Lys-Arg-Arg-Thr-Lys-Ser-Val-Ala-Ala (Pearson et al., 1985) was obtained from Biosearch, Inc.

Nucleotide Analogues. ATP, ADP, AMP, cAMP, GTP, ITP, 1, N^6-ethenoadenosine 5′-triphosphate, CTP, adenosine 5′-(α,β-methylenetriphosphate), adenosine 5′-(β,γ-methylenetriphosphate), adenosine 5′-(β,γ-imidotriphosphate), 2′-deoxy-ATP, 3′-deoxy-ATP, 9-(β-D-arabinofuranosyl)-adenine 5′-triphosphate, 2-chloroadenosine, N^6-methyladenosine, 8-bromoadenosine 5′-triphosphate, and purine riboside were purchased from Sigma Chemical Co. 6-Mercaptoadenosine 5′-triphosphate and 3′-*O*-methyladenosine 5′-triphosphate were purchased from Pharmacia Inc.; adenosine was from Aldrich Chemical Co.; adenosine 5′-*O*-(3-thiotriphosphate) and the *S* isomer of adenosine 5′-*O*-(1-thiotriphosphate) were obtained from Boehringer Mannheim Biochemicals. 8-Azidoadenosine 5′-triphosphate (Czarnecki et al., 1979) was a gift from Boyd E. Haley of the University of Kentucky.

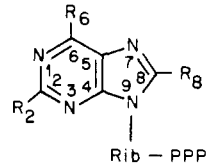
The monophosphate derivatives of N^6-methyladenosine, 2-chloroadenosine, and purine riboside were synthesized by the method of Yoshikawa et al. (1967) and converted to the triphosphates as described by Hoard and Ott (1965). The nucleotide analogues were purified by chromatography over DEAE-cellulose by using a linear gradient of triethylammonium bicarbonate, pH 7.5 (0–400 mM). The stoichiometry of the phosphates was verified by phosphate analysis (Hess & Derr, 1975). The ribose ring opened analogue (**24**) was prepared by periodate oxidation of ATP (Easterbrook-Smith et al., 1976) and subsequent reduction with sodium borohydride; the product was separated from other reagents by chromatography over Sephadex G-10. 2′,3′-*O*-(2,4,6-Trinitrocyclohexadienylidene)-ATP was synthesized and purified by the method of Hiratsuka and Uchida (1973). The purity of all synthesized analogues was determined by thin-layer and/or paper chromatography; common solvent mixtures were used for the development of the chromatograms. The absorption spectra of all nucleotides used in this study were recorded and compared to those previously published. The concentration of each analogue was determined spectrophotometrically at the appropriate wavelength.

Protein Purifications. Calmodulin was prepared from frozen bovine brain (Gopalakrishna & Anderson, 1982). Synapsin I was prepared from fresh bovine brain by modification of the procedure of Ueda and Greengard (1977). The calmodulin-dependent protein kinase was isolated from frozen rat brain essentially as described by McGuinness et al. (1985), except that the steps of high-speed centrifugation and chromatography over Sepharyl S-400 were omitted, and chromatography over hydroxylapatite (0.15–0.45 mM K_2HPO_4, pH 7.5) was added as the last step. The enzyme was at least 90% pure and exhibited a V_{max} of 2.1 μmol min^{-1} mg^{-1} with synapsin I as the substrate.

Measurements of Protein Kinase Activity. The calmodulin-dependent kinase was assayed at 25 °C by measuring the transfer of ^{32}P from [γ-^{32}P]ATP to synapsin I (3.8 μM) or a synthetic peptide substrate (5 μM). The standard assays contained enzyme (0.17 nM, for phosphorylation of the peptide; 0.83–2.5 nM, when synapsin I was used as the substrate), calmodulin (2 μM), [γ-^{32}P]ATP (0.01–0.1 mM at 70–400 Ci/mol), bovine serum albumin (1 mg/mL), glycerol (10%), EDTA (0.1 mM), $CaCl_2$ (0.1 mM), $MgCl_2$ (routinely 10 mM; 30 mM where indicated), dithiothreitol (0.1 mM), and Hepes buffer (60 mM, pH 7.5). Under these conditions kinase activities of 1–3 μmol min^{-1} mg^{-1} were obtained with the two substrates at saturating concentrations of ATP; the K_m and K_i values for ATP did not vary with the substrate used. Incorporation of the radioactivity into synapsin I and the peptide substrate was measured according to Corbin and Reimann (1974) and Roskoski (1983), respectively.

Synapsin I is known as one of the best protein substrates for CaM kinase II; reported kinetic parameters show a range of 0.4–1.2 μM for the K_m (McGuinness et al., 1985; Miller & Kennedy, 1985) and values up to 4.4 μmol min^{-1} mg^{-1} for the specific activity (McGuinness et al., 1985). We found that the ability of synapsin I to serve as a substrate of CaM kinase II decreased significantly at concentrations of $MgCl_2$ above 10 mM; consequently, kinase assays in this study were routinely carried out at 10 mM $MgCl_2$; the concentration of the salt was increased to 30 mM only when high concentrations of ATP analogues were required. It should be emphasized that the inhibitory effect of $MgCl_2$ was not enzyme-directed as it was not observed when syntide 2, a recently recommended peptide substrate of CaM kinase II (Hashimoto & Soderling, 1987), was used in the analysis of the Mg^{2+} effect. Furthermore, the K_m and K_i values for ATP were identical regardless of which substrate or concentration of $MgCl_2$ was employed. Regarding the inhibition of synapsin I phosphorylation, it may be important to note that the phosphorylation of this substrate, but not that of syntide 2, by CaM kinase II also is inhibited by increasing concentrations of NaCl, which suggests that synapsin I may be sensitive to any increases in ionic strength.

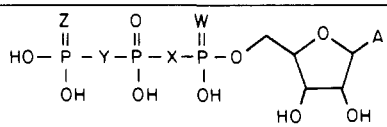
Table I: Inhibition Constants for Base-Modified Analogues of ATP



no.	R_2	R_6	R_8	analogue	K_i (μM)	K_i/K_m
1	H	SH	H		6200[a]	190
2	H	OH	H	ITP	9900[a]	300
3	NH_2	O	H	GTP	5300[a]	160
4	H	H	H		590	18
5	H	$NHCH_3$	H		220	6.7
6	Cl	NH_2	H		110	3.3
7	H	NH_2	Br		460	14
8	H	NH_2	N_3		61	1.8
9				CTP	>50000[a]	
10				etheno-ATP	1300	39

[a] The inhibition constants for these analogues were determined in the presence of 30 mM $MgCl_2$ in order to prevent significant changes in the concentration of Mg^{2+} when millimolar concentrations of nucleotides were required for the experiments. The higher concentration of the salt was not routinely employed because of significant substrate-directed inhibition of the phosphorylation of synapsin I at concentrations of $MgCl_2$ above 10 mM (see Materials and Methods).

Table II: Inhibition Constants for ATP Analogues Modified in the Triphosphate Moiety



no.	Z	Y	X	W		K_i (μM)	K_i/K_m
11	O	CH_2	O	O	AppCp	280	8.5
12	O	NH	O	O	AppNp	190	5.8
13	O	O	CH_2	O	ApCpp	23	0.70
14	S	O	O	O		20	0.61
15	O	O	O	S		23	0.70
16					ADP	24	0.73
17					AMP	740	22
18					cAMP	1100	33
19					Ado	250	7.6

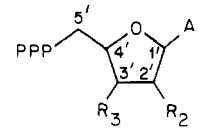
To avoid substrate-directed effects and the time-consuming isolation of synapsin I, a peptide substrate (Pro-Lys-Arg-Arg-Thr-Lys-Ser-Val-Ala-Ala) was used during the latter part of the study. This analogue of the N-terminal 10 residues of glycogen synthase represents one of the best peptide substrates (K_m = 3.5 μM; V_{max} = 11.3 μmol min^{-1} mg^{-1}) for CaM kinase II identified by Pearson et al. (1985).

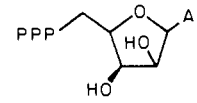
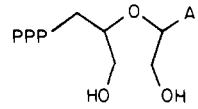
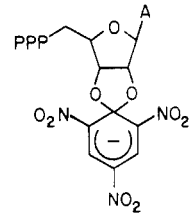
Determination of Inhibition Constants. The inhibition constant for a given analogue of ATP was determined by measuring substrate phosphorylation by [γ-^{32}P]ATP (0.01–0.1 mM) in the absence and presence of the unlabeled analogue. Kinetic analyses of the data according to Lineweaver and Burk (1934) and Hanes (1932) indicated that the inhibition followed a competitive mechanism in all cases. The inhibition constants were obtained by linear regression analysis and averaging of at least two separate experiments. As a control, each analysis of a given analogue was accompanied by a determination of the K_m (33 ± 9 μM) and K_i (34 ± 8 μM) for ATP.

Results and Discussion

The affinity of CaM kinase II for 25 analogues of ATP, containing modifications at the base moiety, ribose ring, or triphosphate chain, was investigated by measuring the transfer of ^{32}P from [γ-^{32}P]ATP to a protein or peptide substrate in competition with the unlabeled analogues. The results were analyzed according to Lineweaver and Burk (1934) and Hanes (1932), which provided the inhibition constant for each analogue (Tables I–III) and indicated a competitive mechanism of inhibition for each compound tested in this study (data not shown).

Table III: Inhibition Constants for Ribose-Modified Analogues of ATP



no.	R_3	R_2	K_i (μM)	K_i/K_m
20	OH	H	39	1.2
21	H	OH	5.1	0.15
22	OCH_3	OH	38	1.2
23			23	0.70
24			270	8.2
25			380	12

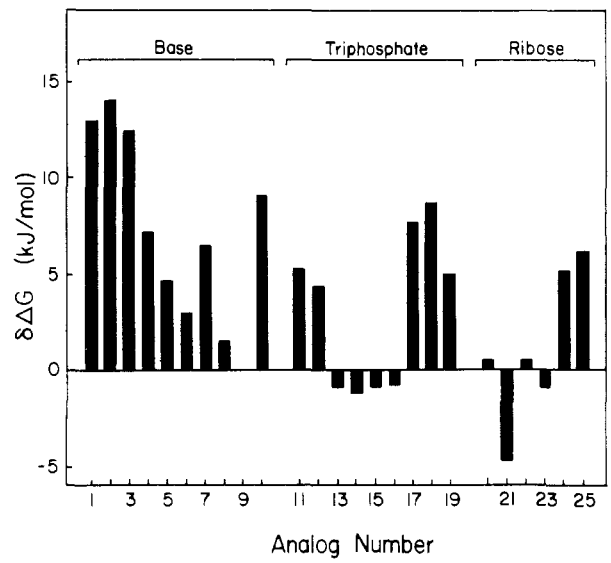


FIGURE 1: Changes in free energy of interaction upon modification of the ATP molecule. $\delta\Delta G$ values were calculated as described in the text. A positive $\delta\Delta G$ value corresponds to a decrease in the affinity of a given analogue relative to ATP, and a negative value indicates an increase. The analogues are listed by the numbers defined in Tables I–III; they are shown in three groups according to the portion of the ATP molecule that was modified.

The inhibition constants of the ATP analogues were compared to the K_m for ATP to identify important determinants for ATP binding to CaM kinase II. In order to estimate the effect of the modifications on the change in free energy of interaction of CaM kinase II with ATP (ΔG = −25.5 kJ/mol), the K_i values for the analogues were compared on a free energy scale (Figure 1); the following equation was used to calculate the corresponding $\delta\Delta G$ values:

$$\delta\Delta G = -RT \ln [K_m(\text{ATP})/K_i(\text{analogue})]$$

Table IV: Comparison of Analogue Specificity of Calmodulin-Dependent Protein Kinase, Phosphorylase *b* Kinase, and Cyclic Nucleotide Dependent Protein Kinases[a]

		CaM kinase (K_m = 33 μM)		phosphorylase kinase (K_m = 43.5 μM)		cGMP kinase (K_m = 7.1 μM)		cAMP kinase (K_m = 3.1 μM)	
no.	analogue	K_i (μM)	K_i/K_m	K_i (μM)	K_i/K_m	K_i (μM)	K_i/K_m	K_i (μM)	K_i/K_m
2	ITP	9900	300	1400	32	880	120	450	150
3	GTP	5300	160	1400	32	770	110	450	150
9	CTP	>50000		>15000		>3500		>3500	
6	2-Cl-ATP	110	3.3	310	7.1	36	5.1	95	31
10	etheno-ATP	1300	39	170	3.9	96	14	52	17
12	AppNp	190	5.8	530	12	230	32	75	24
13	ApCpp	23	0.70	82	1.9	30	4.2	9	3
11	AppCp	280	8.5	330	7.6	660	93	110	35
20	2′-dATP	39	1.2	50	1.1	25	3.5	13	4.2
21	3′-dATP	5.1	0.15	32	0.74	5	0.7	0.61	0.20

[a] The K_i values for the calmodulin-dependent protein kinase were determined in the present study; the K_i values for all other kinases were taken from Flockhart et al. (1984).

A positive value for $\delta\Delta G$ indicates a decrease in the affinity of a given analogue in relation to ATP; a negative value corresponds to an increase in affinity.

Modifications at the Adenine Moiety. CaM kinase II appears to be least tolerant to alterations at the base moiety of ATP (Table I and Figure 1). The affinity of the enzyme decreased by at least 160-fold ($\delta\Delta G \geq 12.5$ kJ/mol) when the adenine ring was replaced by guanine (GTP), inosine (ITP), or cytosine (CTP). Introduction of an SH group at position 6 (**1**) brought about the same reduction in affinity which is consistent with a very strict recognition of the adenine ring and its NH_2 group by CaM kinase II. Because the size of the NH_2 group is similar to that of SH (**1**) and OH (ITP), these dramatic losses in affinity probably are not due to steric problems but involve electronic effects; a less than 7-fold decrease in affinity upon methylation of the amino group (**5**) supports this assumption. Removal of the NH_2 group (**4**) resulted in an 18-fold decrease in affinity ($\delta\Delta G$ = 7.2 kJ/mol), which suggests that hydrogen bonding to this group may be important in binding.

CaM kinase II showed little sensitivity to the introduction of a chloro group at position 2 (**6**; $\delta\Delta G$ = 3.0 kJ/mol) but responded with an almost 40-fold decrease in affinity ($\delta\Delta G$ = 9.1 kJ/mol) to a linking of positions 1 and N^6 via an etheno group (**10**).

Substitutions at position 8 resulted in decreases in the interactions with CaM kinase II (Table I). The effect was not very significant for the introduction of an azido group (**8**; $\delta\Delta G$ = 1.6 kJ/mol) but corresponded to a $\delta\Delta G$ of 6.5 kJ/mol for the brominated derivative (**7**). This decrease in affinity may be a result of steric effects but could also be related to the conformation of the nucleotides at the glycosidic bond. Introduction of the bromo group at position 8 is known to shift the equilibrium from the normal anti conformation of ATP to the syn conformation (Ikehara et al., 1972).

Modifications at the Triphosphate Chain. The K_i values obtained with analogues of ATP modified at the triphosphate moiety are listed in Table II. Interactions of CaM kinase II with the triphosphate chain appear to contribute much less to the free energy of binding of the ATP molecule (ΔG = −25.5 kJ/mol) than the adenine moiety since deletion of the γ-phosphoryl group (ADP) had no effect on the interactions and removal of the complete triphosphate chain (adenosine) corresponded to a $\delta\Delta G$ of only 5.0 kJ/mol (Figure 1). However, results with several analogues indicate that the enzyme is sensitive to changes in the conformation of this moiety: (i) replacement of the β,γ-bridge oxygen with a methylene (**11**) or imido linkage (**12**) significantly decreased interactions, and (ii) the kinase exhibited a lower affinity for AMP and cAMP than observed for adenosine. Additional analogues in Table II contain modifications at the triphosphate chain that are well tolerated by CaM kinase II: the enzyme was insensitive to the replacement of the α,β-bridge oxygen with a methylene group (**13**) and to the exchange of the oxygen of the α- or γ-phosphoryl groups by sulfur (**14** and **15**).

Modifications at the Ribose Ring. The results of Table III and the corresponding comparison of $\delta\Delta G$ values in Figure 1 indicate that modifications at the ribose ring of the ATP molecule are well tolerated by CaM kinase II. Neither the 3′- nor the 2′-hydroxyl group appears to be involved in binding since removal of either group (**20** and **21**) was without adverse effects on the interactions with the enzyme. However, removal of the 3′-hydroxyl group (**21**) increased the affinity of the kinase for this nucleotide significantly ($\delta\Delta G$ = −4.7 kJ/mol), which suggests a close contact between the kinase and the ATP molecule at this particular position. An insignificant change in the interaction was observed with the 3′-methoxy derivative ($\delta\Delta G$ = 0.45 kJ/mol), but introduction of a bulky bridge connecting the 2′- and 3′-positions (**25**) resulted in a 12-fold decrease in affinity ($\delta\Delta G$ = 6.2 kJ/mol).

The importance of the correct conformation of the ribose ring in binding of ATP to CaM kinase II was investigated with **23** and **24**, which indicated that replacement of ribose by the diastereomer arabinose (**23**) had no significant effect but that opening of the ribose ring between positions 2′ and 3′ (**24**) resulted in a loss of 5.2 kJ/mol (Figure 1) in free energy of interaction.

Comparison between ATP-Binding Sites. The use of ATP analogues has been effective in demonstrating distinct differences in the recognition of ATP by a number of ATP-binding enzymes (Hohnadel & Cooper, 1972; Freist et al., 1976a,b; Baydoun et al., 1981; Flockhart et al., 1984) and in predicting structural homology within one family of protein kinases (Flockhart et al., 1984). The similarlity between the ATP specificity observed in the present study and results of comparable analyses for other protein kinases indicates that CaM kinase II is related to phosphorylase *b* kinase, cAMP-dependent protein kinase, and cGMP-dependent protein kinase (Flockhart et al., 1984). Table IV provides a comparison of the results obtained for these protein kinases with a common set of 10 ATP analogues. Quantitatively, the responses of the four kinases toward specific modifications of the ATP molecule were different, but the overall pattern was the same. The following common features are notable for all four of the enzymes: (i) They are most sensitive to changes at, or replacement of, the adenine moiety of ATP. (ii) They are more tolerant of modifications at the triphosphate chain but retain a selective sensitivity to changes in the conformation of this

group. This is demonstrated by the results with **11–13**, which show that replacement of the β,γ-bridge oxygen with a methylene or imido linkage significantly decreased interactions but replacement of the α,β-bridge oxygen with a methylene group was very well tolerated. (iii) The kinases are least sensitive to modifications at the ribose ring. The 2′- and 3′-hydroxyl groups are not essential features; indeed, 3′-dATP demonstrates a higher affinity for these kinases than ATP.

In contrast, a cyclic nucleotide independent protein kinase was found to be almost insensitive to changes at the adenine moiety (Baydoun et al., 1981), hexokinase is intolerant to changes at the ribose ring (Hohnadel & Cooper, 1972), and the 2′ and 3′-hydroxyl groups are necessary for the catalytic action of several aminoacyl-tRNA synthetases (Freist et al., 1976a,b).

The common ATP specificity of the enzymes in Table IV indicates that CaM kinase II belongs to the previously identified family of protein kinases that includes phosphorylase *b* kinase and the cyclic nucleotide dependent protein kinases (Flockhart et al., 1984). Support for this hypothesis was provided, recently, when the deduced primary structures of the α and β subunits of CaM kinase II were shown to exhibit areas of extensive homology with the protein sequence of myosin light chain kinase, the γ subunit of phosphorylase *b* kinase, and the catalytic subunit of cAMP-dependent protein kinase (Hanley et al., 1987; Bennett & Kennedy, 1987).

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