Effects of Autophosphorylation on Casein Kinase II Activity: Evidence from Mutations in the β Subunit†

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Received December 22, 1993; Revised Manuscript Received March 24, 1994®

ABSTRACT: Casein kinase II is a heterotetramer composed of two catalytic (α) and two regulatory (β) subunits. To examine the effects of autophosphorylation of the β subunit on enzyme activity, two mutants of the β subunit from *Drosophila* were constructed in which either Ser_4 or Ser_{2-4} was changed to alanine residues by oligonucleotide-directed mutagenesis and the proteins were expressed in *Escherichia coli*. The wild-type α and individual β subunits present in inclusion bodies were renatured, and the biochemical properties of the reconstituted holoenzymes were examined. Analysis of autophosphorylation revealed that phosphate incorporation was about 0.8 mol/mol of β subunit for the wild type and Ala_4 mutant; Ser_2 and Ser_3 were the major sites of autophosphorylation with some phosphate in Ser_4 as shown by Edman degradation. No autophosphorylation was observed with the Ala_{2-4} mutant. Substitution of alanine for serine residues at positions 4 or 2–4 of the β subunits did not influence the reassociation of the α and β subunits to form holoenzyme, or the function of the β subunit in stimulating catalytic activity or in responding to basic compounds. To measure the effects of autophosphorylation on casein kinase II activity, the wild-type and mutant holoenzymes were preincubated in the presence and absence of ATP, and the rate of phosphorylation was measured with various substrates. In the absence of autophosphorylation, the wild-type, Ala_4, and Ala_{2-4} forms of the holoenzyme displayed similar rates of phosphorylation of glycogen synthase. After preincubation with ATP, the rate of phosphorylation of glycogen synthase by the wild-type and Ala_4 enzymes was inhibited by 30%. No inhibition was observed with the Ala_{2-4} mutant under the same conditions. Similar results were observed with EF-1β and -δ (50–70% inhibition) and calmodulin (20–40% inhibition) with the autophosphorylated wild type and Ala_4 mutant. No effect of autophosphorylation was observed with casein. The data indicate that autophosphorylation of the β subunit can negatively regulate the phosphotransferase activity of casein kinase II with physiological substrates.

Casein kinase II is a multipotential serine/threonine protein kinase which phosphorylates a variety of proteins crucial to the regulation of cellular functions [reviewed in Tuazon and Traugh (1991) and Pinna (1990)]. The enzyme is found in all eukaryotes examined and in various subcellular compartments. *In vitro*, the phosphotransferase activity of casein kinase II is modulated by two distinct groups of compounds. Basic compounds including spermine and polylysine are stimulators of activity, and acidic compounds including heparin and 2,3-bisphosphoglycerate are inhibitors. *In vivo*, the activity of casein kinase II is stimulated by a number of hormones and growth factors (Sommercorn et al., 1987; Ackerman & Osheroff, 1989; Carroll & Marshak, 1989). In yeast, disruption of both genes encoding the α and α′ subunits is lethal, indicating that casein kinase II is essential for the viability of yeast (Padmanabha et al., 1990). EGF-stimulated cell growth is inhibited by introduction of antisense oligonucleotides complementary to the mRNAs of the α and β subunits of casein kinase II (Pepperkok et al., 1991).

Casein kinase II from most sources exists as a stable heterotetramer composed of two α (and/or α′) subunits and two β subunits. As the protein kinase can be dissociated only under denaturing conditions, the α (α′) subunits have been identified as the catalytic subunits using a variety of methods (Cochet & Chambaz, 1983; Hathaway et al., 1981; Lin et al., 1991; Hu & Rubin, 1990). The β subunit is regulatory in nature, stimulating the catalytic activity (Lin et al., 1991; Grankowski et al., 1991; Brinbaum et al., 1992; Jakobi & Traugh, 1992), mediating the interactions with basic effectors (Lin et al., 1991, 1992; Hu & Rubin, 1990; Traugh et al., 1990; Filhol et al., 1991), and participating in substrate recognition/binding (Lin et al., 1991, 1992; Meggio et al., 1992; Filhol et al., 1992).

In vivo, phosphorylation of the α and β subunits has been observed in reticulocytes (Palen & Traugh, 1991) and in chicken bursal lymphoma BK3A cells arrested at mitosis (Litchfield et al., 1992). In human epidermal carcinoma A431 cells, phosphorylation of the β subunit is estimated in response to epidermal growth factor (Ackerman et al., 1990). *In vitro*, the β subunit of purified casein kinase II is autophosphorylated on serine residues. The same sites have been shown to be phosphorylated in A431 cells by phosphopeptide mapping (Litchfield et al., 1991). Autophosphorylation of the α subunit is observed *in vitro* only in the presence of basic polypeptides such as polyarginine, polylysine, histone, and protamine (Palen & Traugh, 1991; Meggio et al., 1983). In addition, the α and β subunits are reported to be substrates for $p34^{cdc2}$ *in vitro* (Litchfield et al., 1991, 1992; Mulner-Lorillon et al., 1990); Ser_{209} has been identified as the site of phosphorylation in the β subunit (Litchfield et al., 1991).

Casein kinase II preferentially phosphorylates serine/threonine residues followed by a stretch of acidic residues on the immediate C-terminal side. Among these acidic residues

† This research was supported by a grant from the U.S. Public Health Service (GM 26738).

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® Abstract published in *Advance ACS Abstracts,* May 1, 1994.

0006-2960/94/0433-6998$04.50/0

(Glu, Asp, or Ser-P), the one located in the third position after the phosphoacceptor serine/threonine is the most critical determinant when analyzed with peptide substrates [reviewed in Tuazon and Traugh (1991) and Pinna (1990)]. All of the identified sequences for the β subunit of casein kinase II contain a region which fulfills this recognition determinant (Saxena et al., 1987; Heller-Harrison & Czech, 1989; Jakobi et al., 1989; Hu & Rubin, 1991; Boldyreff et al., 1991; Maridor et al., 1991). Three serine residues followed by two glutamate residues in the N-terminus of the β subunit (Met-Ser^2-Ser^3-Ser^4-Glu-Glu-) are postulated to be the region of autophosphorylation (Tuazon & Truagh, 1991). This postulation is supported by the observation that the major phosphopeptide generated from the autophosphorylated β subunit contains the first nine amino acid residues of the N-terminus (Litchfield et al., 1991). Questions still remain as to how many serines are actually phosphorylated as well as the role of these sites in the regulation of casein kinase II functions.

In an effort to answer these questions, we constructed two mutants of the β subunit of *Drosophila* by oligonucleotide-directed mutagenesis, in which either Ser_4 or Ser_{2-4} was substituted by alanine residues. The reassociation of these subunits with the catalytic subunit, characterization of the reconstituted activity, and the effects of autophosphorylation on the rate of phosphorylation of physiological substrates were studied.

EXPERIMENTAL PROCEDURES

Materials. The λgt11 plasmids containing cDNAs encoding the α and β subunits of casein kinase II from *Drosophila* were a gift of Dr. Claiborne V. C. Glover, Department of Biochemistry, University of Georgia, Athens, GA. The pET3a plasmids were generously provided by Dr. F. W. Studier, Brookhaven National Laboratory, Upton, NY. Glycogen synthase from rabbit skeletal muscle was a gift of Dr. Balwant Khatra, California State University, Long Beach, CA. EF-1 from rabbit reticulocytes was purified to apparent homogeneity through gel filtration on Bio-Gel A-5m, affinity chromatography on tRNA–Sepharose and fast protein liquid chromatography on Mono Q (Venema et al., 1991). Urea, glutathione (oxidized), dithiothreitol, IPTG (isopropyl thio-β-D-galactoside), PMSF (phenylmethanesulfonyl fluoride), spermine, mixed histone (type II AS), and polylysine hydrochloride (MW 36 500) were obtained from Sigma. Calmodulin (Ca^{2+}-free) was from Ocean Biologics. GDP was purchased from Boehringer Mannheim Biochemicals. The heparin–TSK column was from Tosohaas. Restriction enzymes were obtained from New England Biolabs. The site-directed mutagenesis kit and [γ-^{32}P]ATP (30 Ci/mmol) were from Amersham. The Gene Clean II kit was from Bio 101. The plasmid midi and mini kits were from Qiagen. Sequenase version 2.0 was from United States Biochemical. The Sequelon disks were from Millipore.

Oligonucleotide-Directed Mutagenesis of the β Subunit of Casein Kinase II. Mutants of the β subunit (Ala_4 and Ala_{2-4}) were created by mutagenesis of cDNA encoding the β subunit in M13 phage using the procedure of Taylor et al. (1985). The oligonucleotide for the Ser_4 to Ala_4 change was 5′-TATGAGCAGCGCCGAGGAAG (mismatch underlined), using the M13 derivative containing the wild-type ssDNA as template. The oligonucleotide for the Ser_{2-4} to Ala_{2-4} mutation was 5′-CCATATGGCTGCCGCCGAGG with the Ala_4 M13 derivative ssDNA as template. The locations of the base changes were confirmed by DNA sequencing (Sanger et al., 1977).

Subcloning and Expression of the α and β Subunits of Casein Kinase II. The cDNAs for the wild-type α and β subunits of casein kinase II from *Drosophila* were excised from *Eco*RI sites of the λgt11 phage and subcloned into M13 phage as described elsewhere (Lin & Traugh, 1993). The cDNAs containing the M13 replicative forms of the wild-type α and the wild-type and mutant β subunits were digested by *Nde*I and *Bam*HI and ligated into *Nde*I and *Bam*HI sites in the pET3a plasmid. The mutation sites were reconfirmed by sequencing after transformation. The bacterial strain BL21(DE3) was used as the host for the recombinant plasmids. The expression of the wild-type and mutant subunits was induced by IPTG as described previously (Lin & Traugh, 1993). The bacteria were harvested and disrupted by sonification after IPTG induction for 2 h. The inclusion bodies containing the individual subunits were collected after low-speed centrifugation of the sonicate and stored at −70 °C (Lin & Traugh, 1993).

Reconstitution and Purification of Recombinant Casein Kinase II. To reconstitute the recombinant holoenzyme, the isolated inclusion bodies containing the individual subunits of casein kinase II were solubilized with 8 M urea and 0.3 M dithiothreitol for 2 h at room temperature, diluted with renaturation buffer, and incubated overnight at 10 °C. The reconstituted holoenzyme was purified via a one-step procedure by fast protein liquid chromatography on a heparin–TSK column (Lin & Traugh, 1993). The elution of the enzyme was monitored by assaying for casein kinase activity, and the proteins were visualized by Coomassie blue staining following electrophoresis in 11% polyacrylamide gels containing sodium dodecyl sulfate (Lin et al., 1991).

Assays for Casein Kinase II Activity. The standard assay for phosphotransferase activity of casein kinase II was conducted in 0.070-mL reaction mixtures containing 50 mM Tris-HCl, pH 7.4, 140 mM KCl, 5 mM $MgCl_2$, 5 mg/mL dephosphorylated casein, 140 μM ATP (500 dpm/pmol), and 10–50 units of casein kinase II. Incubation was at 30 °C for 15 min and was terminated by trichloroacetic acid precipitation of casein on filter paper as described previously (Lin et al., 1991). Autophosphorylation of the enzyme was carried out in the absence of exogenous substrates and analyzed by polyacrylamide gel electrophoresis in sodium dodecyl sulfate followed by autoradiography (Lin et al., 1991).

When the effects of autophosphorylation on casein kinase II activity were examined with glycogen synthase (2.7 μg), EF-1 (2 μg), and calmodulin (2.4 μg) as substrates, casein kinase II (13 units) was preincubated in the presence or absence of ATP for 15 min at 30 °C. Following the addition of substrates, the reactions were carried out for 3 min at 30 °C at a final concentration of 140 μM ATP (1000–2500 dpm/pmol). The monovalent salt concentrations of the reaction mixtures were modified slightly to meet the optimal conditions for the different substrates: 100 mM for glycogen synthase and calmodulin, and 70 mM for EF-1. When casein kinase II activity was assayed with calmodulin and EF-1, polylysine (0.1 μM) and GDP (0.5 mM) were added, respectively, to enhance activity. Phosphorylation of the substrates was analyzed by polyacrylamide gel electrophoresis in sodium dodecyl sulfate folloed by autoradiography. ^{32}P incorporated into the individual proteins was quantified by scanning the autoradiogram or counting the gel slices in a liquid scintillation counter. Quantification of phosphorylation into each substrate was repeated 3–6 times in separate experiments. Data shown are representative of these results.

Table 1: Optimal Stimulation of the Catalytic Activity of Casein Kinase II by Wild-Type or Mutated β Subunits[a]

subunits of casein kinase II	^{32}P incorporated into casein	
	cpm	x-fold
α	9455	1.0
$\alpha + \beta$ WT	45519	4.8
$\alpha + \beta$ Ala_4	46990	5.0
$\alpha + \beta$ Ala_{2-4}	53868	5.7

[a] Inclusion bodies containing the α (6.5 μg) and β subunits (11–18 μg) of casein kinase II were solubilized and renatured as described. The β subunits were in 2–3-fold excess as compared to the α subunit. β WT, wild-type β subunit; β Ala_4, β subunit containing the Ala_4 mutation; β Ala_{2-4}, β subunit containing the Ala_{2-4} mutation. The data shown are representative of three separate experiments.

Identification of Autophosphorylation Sites by Edman Degradation. Casein kinase II (wild type and Ala_4, 240 units) was autophosphorylated in a 120-μL reaction mixture with the addition of 0.1 mg/mL soybean trypsin inhibitor. After polyacrylamide gel electrophoresis, the autophosphorylated β subunit was excised from the gel and subjected to trypsin digestion as previously described (Tuazon et al., 1989). The tryptic peptides recovered were lyophilized, redissolved in 20 μL of 50% acetonitrile, and spotted on an arylamine Sequelon disk (1 cm in diameter). Immobilization of the peptides using water-soluble carbodiimide and sequencing by manual Edman degradation were carried out according to the procedure described by Sullivan and Wong (1991). The amount of ^{32}P-labeled phosphate released from each cycle was measured by Cerenkov counting and compared with the microsequence analysis of the β subunits. In some instances, the methionine from the tryptic phosphopeptide was removed by cyanogen bromide treatment (Charbonneau, 1989) prior to Edman degradation.

Protein Quantitation. Protein concentrations were determined by the dye binding method of Bradford (1976) with bovine γ-globulin as a standard or by scanning Coomassie blue stained gels with bovine serum albumin as a standard.

RESULTS

Effects of Mutations of the β Subunit on Reassociation and Stimulation of the Catalytic Activity of Casein Kinase II. Association of the wild-type and mutant forms of the β subunits from *Drosophila* with wild-type α subunits and the ability of the β subunits to stimulate catalytic activity were examined. The wild-type and mutant forms of the β subunit were present only in inclusion bodies following expression in *E. coli*. These subunits were solubilized and renatured with the α subunit according to the procedures described previously; under these conditions, all of the α subunit is renatured to form holoenzyme (Lin & Traugh, 1993). The β subunit has been shown to significantly stimulate the catalytic activity upon association with the α subunit (Hu & Rubin, 1990; Lin & Traugh, 1993; Grankowski et al., 1991; Brinbaum et al., 1992; Jakobi & Traugh, 1992). Therefore, stimulation of catalytic activity was measured as an indication of subunit reassociation. As shown in Table 1, the wild-type β subunit stimulated the catalytic activity by 4.8-fold when renatured with the α subunit. The Ala_4 mutant, in which Ser_4 was substituted by an alanine residue, stimulated the catalytic activity by 5.0-fold, a result similar to the wild-type enzyme. The second mutant, Ala_{2-4}, in which three serine residues (Ser_{2-4}) were substituted by alanine, stimulated the activity by 5.7-fold. A slightly higher stimulation with the Ala_{2-4} mutant was observed consistently in separate experiments.

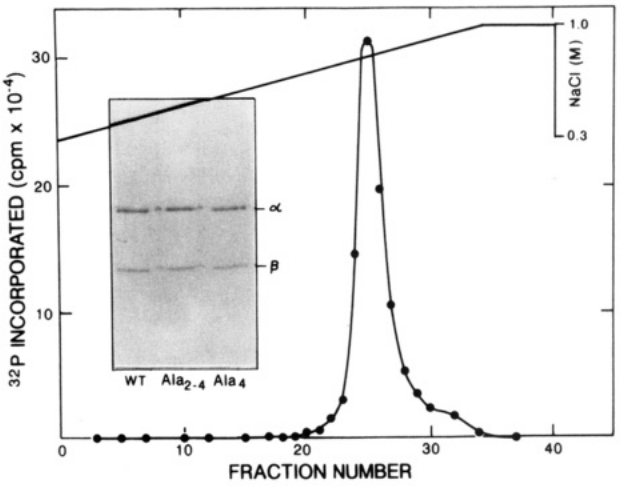

FIGURE 1: Purification of the reconstituted wild-type and mutant forms of casein kinase II on heparin–TSK. The wild-type α and β subunits in inclusion bodies were combined in an approximate 1:2 ratio, solubilized, and renatured, and the reconstituted enzyme was purified by FPLC through a heparin–TSK column. Casein kinase II was monitored by phosphorylation of casein as shown with the wild-type enzyme. Identical activity profiles were obtained with enzymes reconstituted from wild-type α subunits, and β subunits containing Ala_4 and Ala_{2-4} mutations. Inset: the peak fraction from chromatography of each of the three reconstituted enzymes was analyzed by polyacrylamide gel electrophoresis in sodium dodecyl sulfate, and the proteins were visualized by Coomassie blue staining. The positions of the α and β subunits are identified.

To assess directly the association of the mutant β subunits with the wild-type α subunits, the renatured enzymes were purified by fast protein liquid chromatography on a heparin column. A single activity peak eluting at about 800 mM NaCl was obtained with the enzyme reconstituted from the wild-type α and β subunits (Figure 1). Identical activity profiles were obtained with the reconstituted holoenzymes containing the wild-type α and mutant β subunits (data not shown). The protein composition of the peak fractions was analyzed by polyacrylamide gel electrophoresis in sodium dodecyl sulfate followed by Coomassie blue staining (Figure 1, inset). Following elution from the heparin column, the wild-type, Ala_4, and Ala_{2-4} mutants contained only two proteins, the α and β subunits, which were present in an approximate 1:1 molar ratio. The recombinant holoenzymes were purified to apparent homogeneity from the inclusion bodies after the single chromatography step (Figure 1, inset). The results indicated that the mutations at Ser_2 or Ser_{2-4} did not interfere with stimulation of the catalytic activity by the β subunit or with reassociation of the holoenzyme.

Comparison of the Enzymatic Properties of the Mutant and Wild-Type Holoenzymes. The enzymatic properties of the purified holoenzymes containing the mutations were examined and compared to those of the wild-type holoenzyme and the isolated catalytic subunit (Table 2). The two mutated holoenzymes, Ala_4 and Ala_{2-4}, exhibited a specific activity of $(1.2–1.4) \times 10^6$ units/mg with casein, required 150–200 mM KCl for optimal activity, and were stimulated 30–40% by spermine under these assay conditions. All the properties examined were identical to those of the wild-type holoenzyme and distinct from those of the isolated α subunit. Thus, the alanine substitutions did not influence the catalytic activity with casein, the requirement for KCl, or the response to spermine. Taken together, the data indicate correct formation of the holoenzymes and appropriate interactions between the α subunit and mutant forms of the β subunit.

Autophosphorylation of the Wild-Type and Mutant Forms of Casein Kinase II. To examine autophosphorylation of the

Table 2: Comparison of the Properties of Casein Kinase II Containing Wild-Type and Mutated β Subunits[a]

casein kinase II	sp act. (units/mg)	optimal KCl concn (mM)	stimulation by spermine (x-fold)
α	9.0×10^4	50	1.0
$\alpha\beta$ (WT)	1.4×10^6	150–200	1.4
$\alpha\beta$ (Ala_4)	1.2×10^6	150–200	1.3
$\alpha\beta$ (Ala_{2-4})	1.4×10^6	150–200	1.3

[a] The activities of reconstituted casein kinase II containing wild-type α subunit and wild-type [$\alpha\beta$ (WT)] or mutated β subunits [$\alpha\beta$ (Ala_4), $\alpha\beta$ (Ala_{2-4})] were assayed with casein as described under Experimental Procedures. The data for the isolated α subunit were taken from previously published results by Lin et al. (1991). Various concentrations of KCl, ranging from 0 to 300 mM, and spermine (1 mM) were used to examine the effects on casein kinase II.

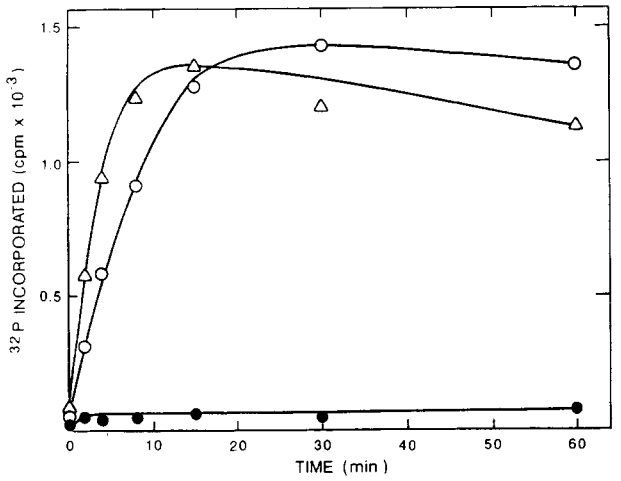

FIGURE 2: Autophosphorylation of the β subunits of wild-type and mutant forms of casein kinase II. The purified wild-type (○), Ala_4 (△), and Ala_{2-4} mutants (●) of casein kinase II (21 μL, 336 units) were incubated with [γ-^{32}P]ATP in a 350-μL reaction mixture, and 50-μL aliquots were removed at the times indicated and analyzed by polyacrylamide gel electrophoresis in sodium dodecyl sulfate followed by autoradiography. The β subunits were excised, and ^{32}P was quantified by liquid scintillation counting. The experiment was conducted 4 times with holoenzymes reconstituted from several preparations of α and β subunits, with identical results.

β subunit, equal amounts of the wild-type and mutant enzymes were incubated with [γ-^{32}P]ATP under standard assay conditions in the absence of exogenous substrate. ^{32}P incorporated into the β subunits was examined over a time period of 60 min. As shown in Figure 2, ^{32}P incorporated into the wild-type β subunit increased with time, reached maximum between 20 and 30 min, and remained level until 60 min (Figure 2). Holoenzyme containing the Ala_4 mutation incorporated phosphate into the β subunit to the same level as the wild-type enzyme, but the rate of autophosphorylation was increased by 30%. Thus, maximal autophosphorylation was reached earlier, between 10 and 15 min. No phosphorylation of the β subunit containing the Ala_{2-4} mutation was detected over the 60-min incubation period. The data indicated that the autophosphorylation site(s) in the Ala_{2-4} mutant, but not in the Ala_4 mutant, was (were) disrupted. Stoichiometric analysis revealed that about 0.8 mol of phosphate was incorporated per mole of β subunit with the wild-type and Ala_4 mutants.

To determine the site(s) of autophosphorylation on the β subunit, the wild type and the Ala_4 mutant of casein kinase II were autophosphorylated with [γ-^{32}P]ATP for 30 min and subjected to manual Edman degradation. The data were compared with the microsequence analysis. As shown in Figure 3, Ser_2 and Ser_3 were highly phosphorylated in both

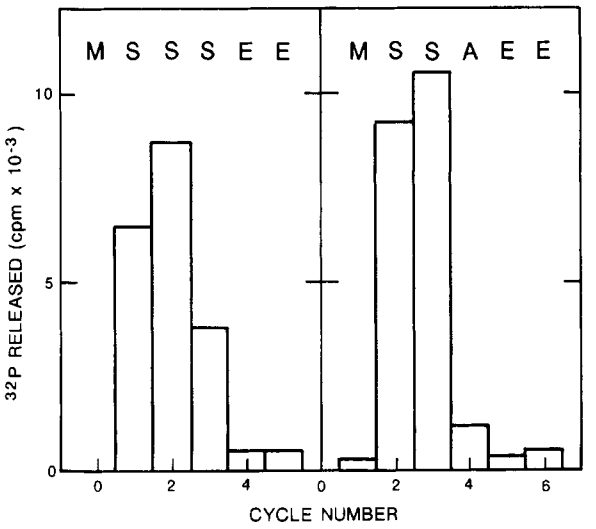

FIGURE 3: Identification of the autophosphorylation sites on the β subunit of casein kinase II. The wild-type and Ala_4 mutants of casein kinase II were autophosphorylated as described, and the tryptic phosphopeptides were subjected to manual Edman degradation as described under Experimental Procedures. The wild-type β subunit was treated with cyanogen bromide prior to analysis. Microsequence analysis was carried out at the Biotechnology Instrumentation Facility at the University of California, Riverside.

subunits, with Ser_3 containing the highest amount. Some phosphate was also observed in Ser_4 of the wild-type β subunit. With shorter phosphorylation times, the amount of phosphate in Ser_4 was reduced as compared with the level of phosphate in the other two sites (data not shown).

To examine the stability of the wild-type and mutant forms of casein kinase II, the enzymes were tested for activity with casein after preincubation with ATP for 15 min. The three enzymes retained similar catalytic activities with casein, suggesting that preincubation did not differentially destabilize the enzymes. In addition, autophosphorylation did not alter the activity of casein kinase II with casein (data not shown).

Effects of Autophosphorylation on the Catalytic Activity of Casein Kinase II with Different Physiological Substrates. To test if autophosphorylation affected the catalytic activity of casein kinase II, wild-type and mutant forms of casein kinase II were preincubated with or without ATP and assayed for activity with glycogen synthase (Figure 4). The wild-type and mutant forms of casein kinase II were preincubated in the absence of ATP (nonautophosphorylation conditions) for 15 min at 30 °C and then assayed for activity by incubation with glycogen synthase for up to 15 min (Figure 4, panel A). All three enzymes displayed a similar rate of phosphorylation of glycogen synthase under these conditions. In contrast, the rate of phosphorylation of glycogen synthase by the wild-type and Ala_4 mutants was reduced by 30%, as compared to the rate with the Ala_{2-4} mutant following preincubation with ATP, as observed in the initial 2–4 min of the reaction (Figure 4, panel B). The rate of phosphate incorporation into glycogen synthase by the wild type and Ala_4 mutant was decreased 30–40% as compared to that with the Ala_{2-4} mutant. Comparison of the activity of casein kinase II under the two different preincubation conditions revealed that autophosphorylation of the wild type and Ala_4 mutant inhibited the rate of phosphorylation of glycogen synthase while the Ala_{2-4} mutant maintained an activity similar to that observed with the nonautophosphorylated enzymes.

To test if the alteration in enzyme activity caused by autophosphorylation was also observed with other physiological substrates, phosphorylation of calmodulin and elongation

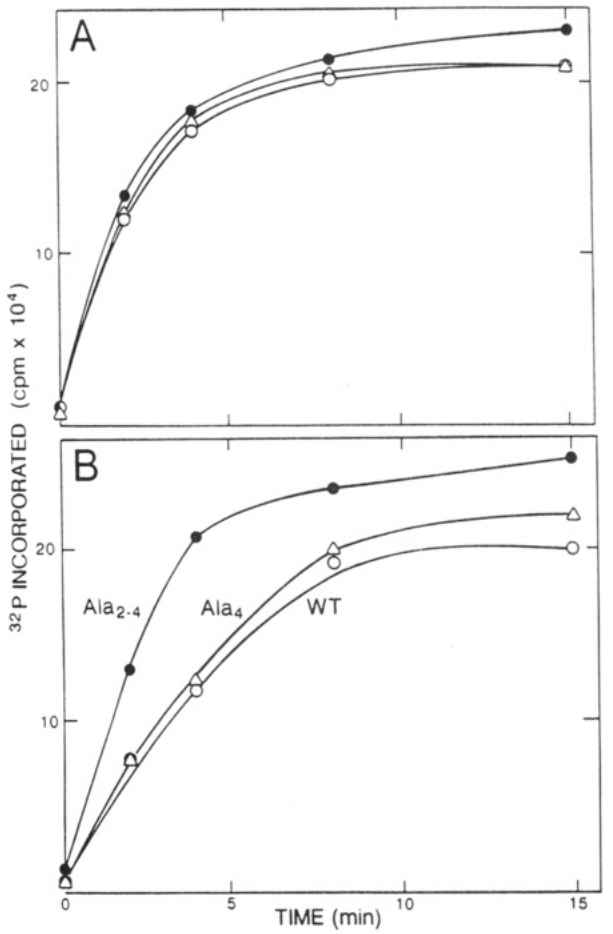

FIGURE 4: Effects of autophosphorylation of casein kinase II on phosphorylation of glycogen synthase. Equal amounts (72 units) of wild-type (○), Ala_4 (△), and Ala_{2-4} mutants (●) were preincubated in the absence (panel A) or presence (panel B) of ATP at 30 °C for 15 min. Protein kinase activity was analyzed with glycogen synthase (15 μg) as substrate in a 220-μL reaction mixture. Aliquots (40 μL) were removed at the times indicated and analyzed by polyacrylamide gel electrophoresis in sodium dodecyl sulfate followed by autoradiography. The radioactivity associated with glycogen synthase was excised from the gel and quantified by liquid scintillation counting. The experiment was carried out 5 times with different preparations of casein kinase II; the data shown are representative of these results.

factor 1 (EF-1) was examined. Kinetically valid conditions were established in order to determine the initial rate of phosphorylation; incorporation was linear for up to 10 min with both substrates.

Significant phosphorylation of calmodulin by casein kinase II has been shown to occur only in the presence of polylysine (Meggio et al., 1987; Nakajo et al., 1988; Lin et al., 1992), and is highly dependent on the ratio of effector to substrate. In order to carry out these studies, the concentration of polylysine required to obtain the optimal rate of phosphorylation of calmodulin was determined. As shown in Figure 5, a ratio as low as 0.01 mol of polysine/mol of calmodulin is sufficient for stimulation, and the rate of phosphorylation is greatly reduced when the ratio is higher than 0.15. For optimal conditions, 0.1 μM polylysine was used (a ratio of 0.02 mol of polylysine/mol of calmodulin) and 3 min of incubation. Autophosphorylation inhibited phosphorylation of calmodulin as shown in a typical experiment in Figure 6, left panel. Phosphorylation of calmodulin was inhibited by 40% and 20%, respectively, when the wild type and Ala_4 mutant were autophosphorylated; no change was observed with the Ala_{2-4} mutant as compared to the nonphosphorylated controls (Table 3).

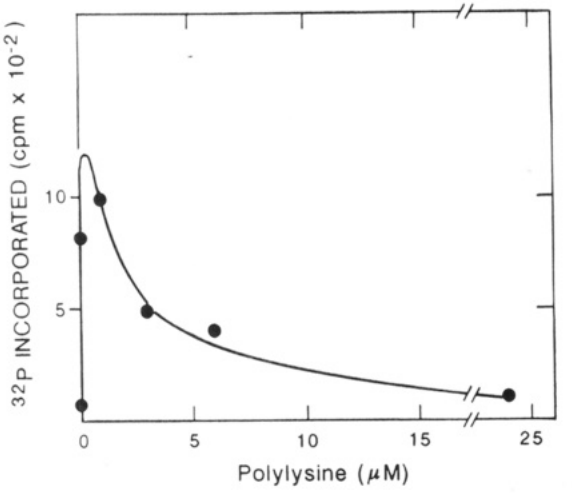

FIGURE 5: Effects of polylysine concentration on phosphorylation of calmodulin by casein kinase II. Calmodulin (5 μg, 12 μM) was incubated for 5 min with 8 units of casein kinase II in the absence and presence of polylysine, as indicated, and analyzed by trichloroacetic acid precipitation.

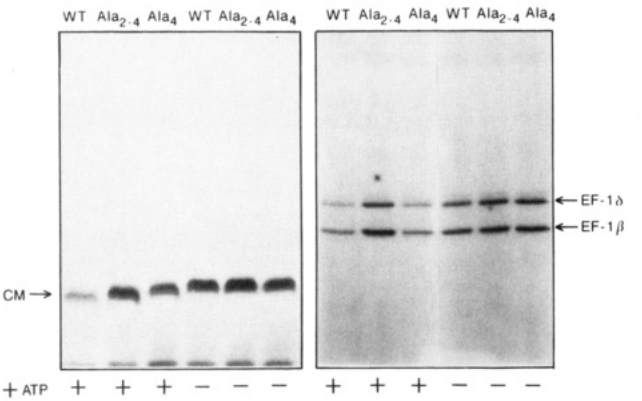

FIGURE 6: Effects of autophosphorylation of casein kinase II on phosphorylation of calmodulin and EF-1. The wild-type, Ala_4, and Ala_{2-4} mutants of casein kinase II (11 units) were preincubated in a volume of 0.009 mL for 15 min in the presence and absence of 140 μM ATP. The autoradiograms are shown. Left panel: Calmodulin (2.4 μg) was added to the preincubated casein kinase II in a final volume of 0.060 mL, and phosphorylation was carried out as described under Experimental Procedures for 3 min. The concentration of ATP in the second assay was 140 μM. The data are representative of those obtained in six different experiments. Right panel: EF-1 (2.0 μg) was preincubated and phosphorylated as described above. The data are representative of those obtained in three different experiments.

Phosphorylation of EF-1 by casein kinase II was carried out in the presence of 0.5 mM GDP, which has been shown previously to stimulate phosphorylation of the β and δ subunits (Palen et al., 1991). As shown in Figure 6, right panel, autophosphorylation diminished the rate of phosphorylation of the β and δ subunits of EF-1. Autophosphorylation of the wild type and Ala_4 mutant inhibited the rate of phosphorylation of EF-1β by 50–60% compared to the nonautophosphorylated counterpart (Table 3). Similar results were obtained with EF-1δ, where phosphorylation was inhibited by 70% after preincubation of the enzymes with ATP. The Ala_{2-4} mutant displayed the same activity whether preincubated with or without ATP. Taken together, the data indicate that autophosphorylation of the β subunits of casein kinase II inhibited protein kinase activity as measured by a decreased rate of phosphorylation of glycogen synthase, EF-1β and -δ, and calmodulin.

Table 3: Quantification of Effects of Autophosphorylation of Casein Kinase II on Activity[a]

	$\alpha\beta$ (WT)			$\alpha\beta$ (Ala_4)			$\alpha\beta$ (Ala_{2-4})		
substrate	+ATP	−ATP	ratio	+ATP	−ATP	ratio	+ATP	−ATP	ratio
glycogen synthase	10000	14600	0.7	10000	15000	0.7	16800	15800	1.1
EF-1β	0.25	0.48	0.5	0.32	0.73	0.4	0.78	0.67	1.2
EF-1δ	0.11	0.39	0.3	0.20	0.58	0.3	0.50	0.59	0.9
calmodulin	2590	4589	0.6	4376	5595	0.8	4419	3865	1.1

[a] The wild-type and mutant forms of casein kinse II were preincubated with and without ATP for 15 min at 30 °C and assayed with glycogen synthase (2.7 μg), EF-1 (2 μg), and calmodulin (2.4 μg) for 3 min. ^{32}P incorporation was quantified by counting the gel slices (glycogen synthase and calmodulin) or scanning the autoradiogram (EF-1). The ratio is +ATP/−ATP.

DISCUSSION

Two mutants of the β subunit of casein kinase II, with alanine replacing serine residues at positions 4 and 2–4, were used to analyze the effects of autophosphorylation on protein kinase activity. Serine residues 2 and 3 are the major sites of autophosphorylation. With the wild-type β subunit, a lower level of phosphate is incorporated into Ser_4, and can be correlated with longer incubation times. The β subunit containing the Ala_{2-4} triple mutation is not autophosphorylated, indicating all the autophosphorylation sites are disrupted in this mutant.

The enzymes reconstituted from the wild-type α and mutant forms of the β subunits show that substitution of alanine residues at position 4 or positions 2–4 does not affect reassociation of the holoenzymes, stimulation of the catalytic activity of the α subunit by the β subunit (about 5-fold), stimulation of activity in response to spermine, the salt optimum for activity, and the rate of phosphorylation with casein. These data indicate that formation of the holoenzyme results in proper interactions between the wild-type α and mutant β subunits and does not cause dramatic changes in subunit interactions. In agreement with our results, studies by Boldyreff et al. (1992) showed that a deletion of four residues from the N-terminus of the β subunit did not influence reassembly of the β subunit with the α subunit or stimulation of catalytic activity by the β subunit; other enzymatic properties were not examined.

Examination of the catalytic activity with various substrates has been carried out in multiple experiments following preincubation of the enzymes with or without ATP. The nonphosphorylated wild-type and mutant enzymes display similar rates of phosphorylation of glycogen synthase. After preincubation with ATP, the rate of phosphorylation of glycogen synthase decreases with the wild type and the Ala_4 mutant; no change in activity is observed with the autophosphorylation defective mutant. Similar results are observed with EF-1β and -δ and calmodulin. Autophosphorylation of the wild type and Ala_4 mutant is shown to decrease the rate of phosphorylation of glycogen synthase by 30–40%, EF-1β and -δ by 50–70%, and calmodulin by 20–40% as compared to the nonautophosphorylated enzymes. Thus, phosphate incorporated into the autophosphorylation site(s) negatively regulates the rate of phosphorylation with the physiological substrates tested, but does not alter the rate of phosphorylation of casein.

Some natural compounds have been shown to have the potential to function as physiological modulators of casein kinase II in different cell types. Spermine and spermidine, at physiological concentrations for reticulocytes, stimulate the activity of casein kinase II by 2.5-fold (Hathaway & Traugh, 1984a), while 2,3-bisphosphoglycerate inhibits the activity of casein kinase II at concentrations equivalent to fluctuating intracellular levels in red blood cells (Hathaway & Traugh, 1984b).

Recent observations suggest that posttranslational modification by phosphorylation, as shown for many other protein kinases, might also play a role in regulating the activity of casein kinase II. Treatment of human carcinoma A431 cells with epidermal growth factor resulted in an increased phosphorylation of the β subunit and a concomitant increase in protein kinase activity with synthetic peptide as substrate (Ackerman et al., 1990). Alkaline phosphatase treatment of casein kinase II from the EGF-stimulated cells significantly reduced the apparent V_{max} with the peptide substrate. The authors suggested that phosphorylation at a site other than the autophosphorylation site(s) was responsible for the observed stimulation of casein kinase II activity. Phosphorylation of the α and β subunits was increased when chicken bursal lymphoma BK3A cells were arrested at mitosis by nocodazole treatment (Litchfield et al., 1992). The increased phosphorylation on both subunits was localized to peptides that were phosphorylated by $p34^{cdc2}$ kinase *in vitro*, suggesting that casein kinase II may be regulated in a cell cycle-dependent manner.

Our studies show definitively for the first time that autophosphorylation of the β subunit negatively regulates the catalytic activity of casein kinase II with physiological substrates. Since the same sites are phosphorylated *in vivo*, as shown by phosphopeptide mapping (Litchfield et al., 1991), casein kinase II activity appears to be regulated by autophosphorylation *in vivo*. Since most substrates of casein kinase II are highly phosphorylated and phosphate at these sites has a slow turnover rate, autophosphorylation of the β subunit in the absence of available substrate would function to inhibit the activity of casein kinase II to diminish nonspecific phosphorylation events.

ACKNOWLEDGMENT

We thank the following individuals for their generosity: Dr. C. V. C. Glover for providing cDNAs for the α and β subunits of casein kinase II, Dr. F. W. Studier for the pET3a plasmids, Dr. B. Khatra for glycogen synthase, Yu-Wen Edith Chang for providing purified EF-1, Mr. Werner Witke for the initial subcloning of the cDNAs for casein kinase II, and Dr. Polygena Tuazon for carrying out the sequence analysis and for many helpful discussions.

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