ATP Nucleotidylation of Creatine Kinase

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ABSTRACT: Creatine kinase (CK) will autoincorporate radiolabel from [γ^{32}P]ATP and has thus been reported to be autophosphorylated. Also, in contrast to normal brain enzyme, CK in Alzheimer-diseased brain homogenate shows greatly decreased activity, abolished photolabeling with [^{32}P]8N_3ATP, and no detectable autoincorporation of radiolabel by [γ^{32}P]ATP. Surprisingly, our studies with both human brain and purified CK showed that [α^{32}P]ATP, [γ^{32}P]ATP, [α^{32}P]ADP, [2,8H^3]ATP, [γ^{32}P]2′,3′-*O*-(2,4,6-trinitrophenyl)-ATP, and [γ^{32}P]benzophenone-γATP all autoincorporate radiolabel into CK with good efficiency. This demonstrates that the γ-phosphate and the 2′ and 3′ hydroxyls are not involved in the covalent linkage and that all three phosphates, the ribose and base of the ATP molecule are retained upon autoincorporation (nucleotidylation). Treatment with $NaIO_3$ to break the 2′−3′ linkage effected total loss of radiolabel indicating that nucleotidylation resulted in opening of the ribose ring at the C1′ position. Nucleotidylation with increasing [α^{32}P]ATP at 37 °C gives an approximate $k_{0.5}$ of 125 μM and saturates at 340 μM nucleotide. Modification of 8−10% of the copy numbers occurs at saturation, and CK activity is inhibited to approximately the same degree. Low micromolar levels of native substrates such as ADP, ATP, and phosphocreatine substantially reduce [α^{32}P]ATP nucleotidylation. In contrast, AMP, GTP, GMP, NADH, and creatine did not effectively reduce nucleotidylation. When [α^{32}P]ATP-nucleotidylated or [α^{32}P]8N_3-ATP-photolabeled CK is treated with trypsin a single, identical radiolabeled peptide (V^{279}-R^{291}) is generated that comigrates on reverse phase HPLC and Tris-tricine electrophoresis. Nucleotidylation into this peptide was prevented 86% by the presence of ATP. We conclude that CK is nucleotidylated within the active site by modification at the C1′position and that autophosphorylation of this enzyme does not occur.

Creatine kinase[1] (ATP:creatine N-phosphotransferase, EC 2.7.3.2) is a key enzyme in eukaryotic energy metabolism that catalyses the regeneration of ATP from phosphocreatine and ADP. An earlier study of aberrant nucleotide interacting proteins in Alzheimer disease (AD) brain versus control brain an experiment was done looking for changes in adenylylation. An initial observation showed that when control human brain homogenate was incubated with [α^{32}P]ATP about five proteins incorporated significant radiolabel stable to SDS-PAGE conditions. One of these proteins was identified as creatine kinase (CK) (*1*). However, in AD brain homogenate, CK was the only protein of the five that showed diminished autoincorporation of radiolabel with [α^{32}P]ATP (an average 87% decrease). CK in AD brain homogenates was also shown to have greatly decreased activity, not to photolabel, and to be abnormally partitioned into the particulate fraction (*1*). Further, experiments showed that purified CK also was unexpectedly radiolabeled with [α^{32}P]ATP and [α^{32}P]ADP.

Enzymes can be covalently modified using nucleotide triphosphate by phosphorylation, adenylylation or *nucleotidylation*. Hemmer and Quest showed that CK would incorporate radiolabel on incubation with [γ^{32} P]ATP and proposed that CK was an autophosphorylated enzyme (*2*, *3*). In addition, CK has been proposed to be posttranslationally modified by phosphorylation (*3*−*6*). Others have incubated CK with [γ^{32}P]ATP and showed that the resultant radiolabeled peptide was identical to one identified as an active site peptide by photoaffinity labeling with [^{32}P]8N_3-ATP (*7*−*9*).

In this report experiments were done to demonstrate that CK is neither an autophosphorylated enzyme as previously reported nor is it adenylylated. Rather, CK autoincorporates the entire ATP or ADP molecule in a nucleotidylation process within the ATP binding site by a process that involves modification through the 1′ carbon by a residue, most likely C^{282}, within the active site peptide identified by photoaffinity labeling.

MATERIALS AND METHODS

Materials. CK isozymes (MM, MB, BB) and trypsin were from Sigma Chemical Company (St. Louis, MO). Protein molecular weight standards were obtained from Bio-Rad (Hercules, CA). All other reagents were purchased from either Aldrich Chemical Company (St. Louis, MO) or Sigma and were of analytical grade. Brain samples were homogenized in a Potter-Elvenhjem glass homogenizer at 4 °C to give a final suspension of 7 mg/mL protein in 20 mM Tris-HCl, pH 7.1/20% glycerol as previously reported (*1*).

Radiochemicals. 8N_3ATP, [α^{32}P]8N_3ATP, [α^{32}P]8N_3ADP, [α^{32}P]ATP, and [^{32}P]8N_3ADP were obtained from Affinity

[1] Abbreviations: CK, creatine kinase; CKBB, creatine kinase from brain; CKMM, creatine kinase from muscle; AD, Alzheimer's disease; 8N_3ATP, 8-azidoadenosine-5′-triphosphate; 2N_3ATP, 2-azidoadenosine-5′-triphosphate; ATP-γ-BP, ATP with *N*-((4-benzoyl)phenylmethyl)-phosphoramide linkage to the γ-phosphate; TNP-ATP, [γ^{32}P]2′,3′-*O*-(2,4,6-trinitrophenyl)-ATP; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; PSM, protein-solubilizing mix.

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Labeling Technologies, Inc. (Lexington, KY). The specific activity was between 10 and 20 mCi per micromole. [2,8 ^{3}H]ATP (specific activity 16.1 mCi/μmol) was purchased from Moravak Biochemicals (Brea, CA). [γ^{32}P]ATP-γBenzophenone ([γ^{32}P]ATPγBP) and 2′,3′-*O*-(2,4,6-trinitrophenyl)-[γ^{32}P]ATP ([γ^{32}P]TNP-ATP) were synthesized as described previously (*10*, *11*, *28*).

CK Activity Assay. CK was assayed using the CK diagnostic kit (DG147-UV) from Sigma as previously reported (*1*).

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE). Following solubilization samples were subjected to discontinuous SDS-PAGE using a 6−10% gradient separating gel with a 4% stacking gel using the Tris-glycine buffer system as described previously (*12*). Electrophoresis was carried out at 35 mA, constant current, for 3−4 h until the dye front eluted and then stained for at least 1 h with Coomassie brilliant blue-R (0.15% Coomassie BBR-250, 25% 2-propanol, 10% acetic acid) and destained overnight (10% 2-propanol, 5% acetic acid). Gels were dried on a slab gel dryer and subjected to autoradiography for 12−48 h.

Nucleotidylation of Purified CK. Nucleotidylation of purified CK (typically 5 μg) using [α^{32}P]ATP, [γ^{32}P]ATP, or [α^{32}P]ADP was done with modifications as indicated at either 22 or 37 °C in Eppendorf tubes that contained a reaction mixture of 1.0 mM $MgCl_2$, 10 mM sodium phosphate, pH 6.8, and varying concentrations of [α^{32} P]-ATP, [γ^{32} P]ATP, or [α^{32}P] ADP (specific activity of about 10 mCi/μmol), in a final volume of 60 μL. Ultrafiltration, where reported, was done using a 30 kD molecular weight cutoff membrane (Amicon 30, Millipore, MA) to remove unbound nucleotide. Nucleotidylation mixtures were incubated for 1 h with the nucleotide unless otherwise reported. Mixtures were directly solublized in protein-solubilizing mixture (PSM) consisting of 5 M urea, 0.125 mM Tris-HCl, pH 6.8, 0.2% SDS, 5% 2-mercaptoethanol, and 0.05% Bromophenol Blue) and analyzed by SDS-PAGE. Alternatively, the reaction was terminated by the addition of ice-cold 7% trichloroacetic acid (TCA) and centrifuged. The supernatant was drawn off, and the precipitate was solubilized in PSM and analyzed by SDS-PAGE. Detection of ^{32}P incorporation was accomplished either by autoradiography followed by quantification on an AMBIS 4000 optical density detector. Alternatively, dried SDS-PAGE gels were scanned directly with an AMBIS 4000 radioanalytic detector (AMBIS, Inc., San Diego, CA) or labeled bands were excised from the dried gel and ^{32} P or ^{3}H incorporation was quantified by liquid scintillation counting.

Isolation of Tryptic of CK. Photolabeled tryptic peptides of CK were generated and isolated as described previously (*8*). For photolabeling, 500 μg of CK was incubated in 10 mM Tris-HCl, pH 7.3, 0.5 mM $MgCl_2$ for 30 s with 50 μM [α^{32}P]8N_3ATP at 4 °C and then photolyzed in the presence of UV light at 254 nm for 60 s with a handheld 254 nm Mineralight US-11 UV lamp (I = 4600 μW/cm^2). For nucleotidylation, 500 μg of CK was labeled with 225 μM [α^{32}P]ATP in the presence of 1 mM $MgCl_2$, 10 mM Tris-HCl at pH 8.5, 37 °C with 60 min incubation. Both photolabeling and nucleotidylation reactions were terminated by the addition of ice-cold 7% TCA. After removal of the supernatant, the precipitate was resolubilized in 1% ammonium acetate/1.2 M urea, pH 8.0 and incubated with 15% w/w trypsin for 24 h at room temperature.

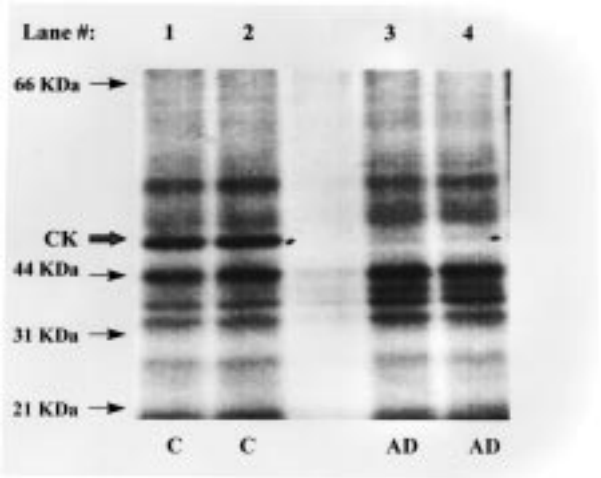

FIGURE 1: An autoradiogram made from a 6−10% SDS-PAGE gel on which brain supernatant proteins were separated after nucleotidylation with [α ^{32}P]ATP as described in methods. Lanes 1 and 2: control brain samples. Lanes 3 and 4: AD brain samples.

Reverse Phase HPLC. The radiolabeled peptides were separated on an Aquapore RP-300 C8 column (250 × 4.6 mm) using the following gradient: 0% solvent B at 0 min, 0% solvent B at 10 min, 30% solvent B at 30 min, 60% solvent B at 60 min, and 100% solvent B at 70 min (solvent A, 0.1% trifluoroacetic acid/50 μM $AlCl_3$; solvent B, 80% acetonitrile/0.1% trifluoroacetic acid). The flow rate for the entire gradient was 0.5 mL/min, and fractions containing radiolabel were analyzed on an Applied Biosystems 477A protein sequencer (*8*).

RESULTS

Decreased [α^{32}P]ATP Auto-radiolabeling of CK in AD Brain Homogenates. Control and AD brain homogenates were incubated with [α^{32}P]ATP at 37 °C for 1 h and ^{32}P incorporation into CK was determined as described in Materials and Methods. The results shown in the autoradiogram in Figure 1 were consistently observed in over 40 samples. CK in control homogenates covalently incorporated [α^{32}P]ATP (lanes 1 and 2) whereas the AD homogenates (lanes 3 and 4) usually showed no detectable ^{32}P incorporation into CK. Identification of the protein as CK was confirmed by Western blot analysis, peptide analysis, and comigration with purified rabbit brain CK (David et al., 1998). Further, the copy number of CK was not markedly lower in AD brain. However, in contrast to controls, AD CK was over 95% partitioned into the particulate fraction. Thus, CK of AD brain homogenate showed a major decrease in [α^{32}P]ATP auto-incorporation versus control brain and was abnormally partitioned.

Saturation of [α^{32}P]ATP Auto-incorporation at 37 °C. Auto-incorporation of [α^{32}P]ATP into purified CKMM (5μg) at pH 6.8 and 37 °C showed saturation at approximately 340 μM [α^{32}P]ATP with an apparent k_d of 125 μM as shown in Figure 2. To determine the efficiency of radioincorporation, the radiolabeled bands were excised from the gel and counted by liquid scintillation. At saturation concentrations of [α^{32}P]-ATP, 8−9% of the copy numbers were modified by auto-incorporation. It should be noted that this is the minimum

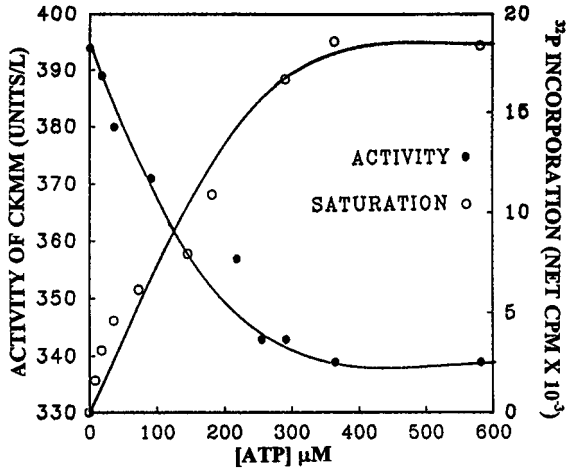

FIGURE 2: Correlation of saturation of [α^{32}P]ATP incorporation into CKMM by nucleotidylation (open circles) with inhibition of CK activity (solid circles). CK was nucleotidylated with varying concentrations of [α^{32}P]ATP, and ^{32}P incorporation was determined after SDS-PAGE as described in methods. For inhibition, the CK was nucleotidylated with identical ATP concentrations and the CK activity was assayed as described in Materials and Methods.

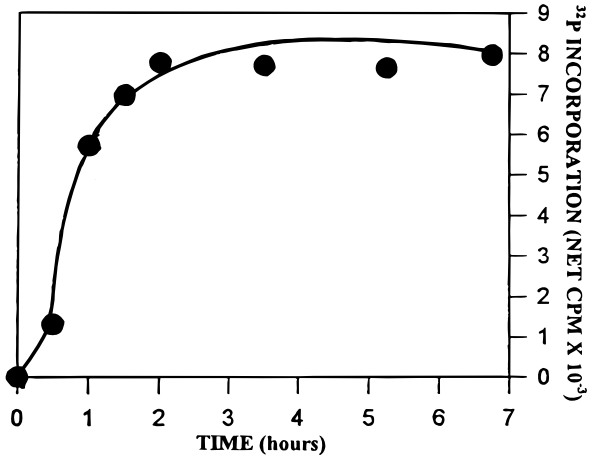

FIGURE 3: Saturation of [α^{32}P]ATP incorporation into CKMM as a function of time. CK was nucleotidylated at 37 °C, pH 6.8 in 10 mM phophate with 250 μM [α^{32}P]ATP for various lengths of time as described in Materials and Methods. ^{32}P incorporation was determined after subjecting the reaction to SDS-PAGE as described in Materials and Methods.

efficiency since the stability of auto-incorporated radiolabel to SDS-PAGE and other workup conditions is unknown.

Time Course of [α^{32}P]ATP Nucleotidylation at 37 °C. CKMM was incubated with 250 μM [α^{32}P]ATP at 37 °C and pH 6.8. Aliquots were withdrawn at various time intervals, and ^{32}P incorporation was determined as described in Materials and Methods. Saturation of ^{32}P incorporation occurred within 2 h at 37 °C as shown in Figure 3.

Effect of Temperature. CKMM (5 μg) or human control brain homogenate (27 μg) was incubated 2 h with 300 μM [α^{32}P]ATP at 4, 22, and 37 °C, and ^{32}P incorporation was quantified as described in Materials and Methods. ^{32}P incorporation showed a 77% increase from 4 to 22 °C and a 55% increase from 22 to 37 °C for purified CKMM. CK nucleotidylation in the brain homogenates showed a 5-fold increase when the temperature was increased from 4 to 22 °C and a 60% increase from 22 to 37 °C.

Auto-incorporation of [α^{32}P]ATP, [α^{32}P]ADP, and [γ^{32}P]-ATP into CK. An initial control experiment unexpectedly

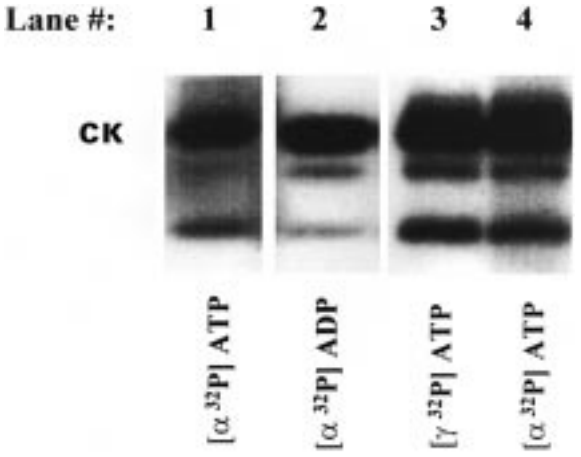

FIGURE 4: Autoradiograms made from two 6−10% SDS-PAGE gels on which purified CKMM was separated after nucleotidylation, as described in Materials and Methods, with the following nucleotides. Gel 1: lane 1, [α^{32}P]ATP (s.a. = 10 mCi/μM); lane 2, [α^{32}P]-ADP (s.a. = 10 mCi/μM). Gel 2: lane 3, [γ^{32}P]ATP (s.a. = 15 mCi/μM); lane 4, [α^{32}P]ATP (s.a. = 15 mCi/μM). CK is the top band.

showed that [α^{32}P]ADP auto-incorporated into CK in human brain. To determine whether the autoincorporation observed was adenylylation, phosphorylation, or nucleotidylation, purified rabbit muscle CK (5 μg) was incubated at pH 6.8 and 37 °C with 250 μM of either [α^{32}P]ADP, [α^{32}P]ATP, or [γ^{32}P]ATP of almost equal specific activity, and ^{32}P incorporation was determined as described in Materials and Methods. Normal results are shown in Figure 4 where [α^{32}P]-ADP (lane 2) incorporated with a 28% greater efficiency than [α^{32}P] ATP (lane 1). Under the same conditions, [γ^{32}P]-ATP (lane 3) and [α^{32}P]ATP (lane 4) incorporated ^{32}P label with similar efficiency (within 5%). These experiments were repeated a minimum of three times with consistent reproducible results.

Auto-incorporation Studies with [γ^{32}P]2N$_3$ATP, [γ^{32}P]-TNP-ATP, [γ^{32}P]ATPγBP, and [2,8H^3]ATP. Incubation of CK with these probes was done to define the site of attachment of the nucleotide to CK and to determine if the base remained attached to the enzyme after modification. The results are shown in Figure 5. [γ^{32}P]2N$_3$ATP (lane 1), [γ^{32}P]TNP-ATP, (lane 2), [γ^{32}P]ATPγBP (lane 3), [2,8 ^{3}H]-ATP (lane 4), and [α^{32}P]8N$_3$ATP, each at a concentration of 300 μM, were separately incubated with CKMM in the absence of activating UV light at pH 6.8 for 60 min ^{32}P incorporation into CK was determined as described in Materials and Methods. ^{3}H incorporation was detected by enhanced autoradiography and liquid scintillation counting. The results show that attachment was not hindered by modification of the 8- or 2-base position (2 or 8N$_3$ATP) or blockage of the 2′-3′ hydroxyl ribose positions (TNP-ATP). Also, the γ-phosphate is not the site of attachment because [α^{32}P]8N$_3$ADP and [γ^{32}P]ATPγBP auto-incorporate with similar efficiencies. Further, the auto-incorporation of radiolabel from [2,8 ^{3}H]ATP showed that the base remained attached.

Specificity for CK. In the control brain homogenate only five proteins significantly auto-incorporate [α^{32}P]ATP, one of them being CK. Only CK showed a significant decrease in auto-incorporation in AD brain homogenates. To determine if the nucleotidylation of CK was a nonspecific and

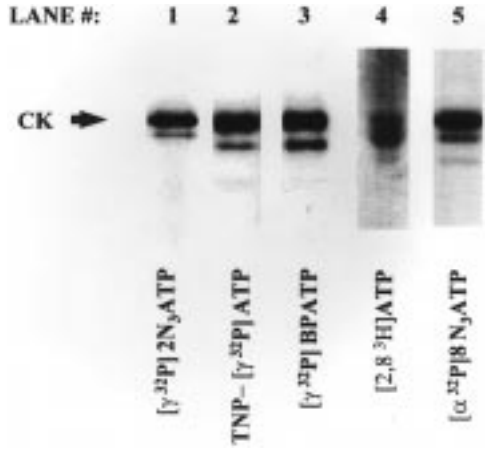

FIGURE 5: An autoradiogram made from a 6−10% SDS-PAGE gel on which purified CKMM was separated after nucleotidylation, as described in Materials and Methods, with the following nucleotide analogues: lane 1, [γ^{32}P]2N_3ATP; lane 2, [γ^{32}P]TNP-ATP; lane 3, [γ^{32}P]ATPγBP; lane 4, [2,8 ^{3}H]ATP (s.a. = 16.1 mCi/μM); lane 5, [α^{32}P]8N_3ATP.

Table 1: The Effect of Various Nucleotides at 400 μM on the Nucleotidylation of CKMM with 200 μM [α^{32}P]ATP at pH 6.8 and 22 °C

	AMP	ADP	ATP	GMP	GTP	NAD	PCr
% of control	90	35	52	73	76	86	57

spurious phenomenon only seen in brain tissue, several ATP binding enzymes were incubated with 200 μM [α^{32}P]ATP at pH 6.8 and ^{32}P incorporation was determined as given above. Eight micrograms each of actin, glutamine synthetase, myokinase, fructose-6-phosphokinase, pyruvate kinase, hexokinase, 3-phosphoglyceric phosphokinase, and CKMM were tested. CK auto-incorporated radiolabel greater than 20 times the next highest protein, and most showed no radiolabel incorporation. Therefore, CK was the only ATP binding enzyme of the group to significantly nucleotidylate (data not shown).

Saturation of Nucleotidylation at 22 °C and Isozymes Specificity. CKMM was nucleotidylated with increasing [α^{32}P]ATP (320 μM maximum) at pH 6.8 at 22 °C. ^{32}P incorporation was detected and quantified as given above. Saturation of ^{32}P incorporation occurred at approximately 195 μM [α^{32}P]ATP with an apparent k_d of 57 μM at pH 6.8 (see Table 1). Under these conditions, CKBB showed a saturation of ^{32}P incorporation at approximately 190 μM [α^{32}P]ATP with an apparent k_d of 35 μM at pH 6.8, Table 2. The data are consistent with only slight differences between the isozymes.

Specific reduction of nucleotidylation of CK with [α^{32}P]-ATP by native substrates. CKMM (5 μg) was incubated at 22 °C for 60 min with 400 μM nonradioactive compound as described in Materials and Methods. After the preincubation unbound nucleotide was removed by ultrafiltration (see Materials and Methods) and 200 μM [α^{32}P]ATP was added to the CK and incubated for an additional 60 min under the same conditions. ^{32}P incorporation was detected and quantified, and the results are shown in Table 1. ADP reduced nucleotidylation most effectively followed by ATP and creatine phosphate. GTP, AMP, and NAD^+ had much less effect consistent with their weak interactions with CK.

Effect of Creatine on Nucleotidylated CK. The forward reaction of CK, i.e., the phosphorylation of creatine, is favored at pH 9.0. There was the unlikely possibility that the radiolabeled CK−[γ^{32}P]ATP complex contained a [^{32}P]-phosphorylated intermediate that could be transferred to creatine. Therefore, [^{32}P]CK was formed by nucleotidylation at pH 9.0 at 37 °C with a 60 min incubation in the presence of 150 μM [γ^{32}P]ATP. The reaction was stopped by ultrafiltration to remove unreacted [γ^{32}P]ATP. The [^{32}P]CK complex was washed twice with 10 mM Tris-HCl buffer, pH 9.0 and then incubated at pH 9.0 in the presence or absence (control) of 18 mM creatine for various time periods. Aliquots were withdrawn at the given time intervals, ^{32}P incorporation into creatine was determined by thin-layer chromatography, and the extent of radiolabel in CK was determined as previously reported (*8*). No [^{32}P]creatine phosphate was detected. The covalent CK−[γ^{32}P]ATP bond formed was inherently unstable at 37 °C, pH 9.0, and the control showed a linear 15% loss in radiolabel over 60 min (see Table 3). In comparison to the control, CK−[γ^{32}P]ATP complex incubated with creatine registered a linear 24% loss in radiolabel over 60 min which is to slow to represent a covalent intermediate in the transphosphorylation reaction.

Effects of a Putative Transition State Complex on Autoincorporation into CK. MgADP−creatine−NO_3 has been described as a transition state complex with very high affinity for the active site of CK. Therefore, a MgADP−creatine−NO_3 complex with CK was formed using 600 μM ADP, 5 mM $NaNO_3$, 20 mM creatine, 1 mM magnesium acetate, in 20 mM buffer (Bis-Tris, pH 7.0) and incubated for 60 min at 37 °C. Similar controls were done using creatine, MgADP, and both creatine and MgADP. After the preincubation to form complex, 100 μM [α^{32}P]ATP was added and incubation was continued for an additional 60 min at 37 °C. Ionic strength was kept constant using 5 mM sodium acetate. The reaction was terminated, and the level of ^{32}P incorporation was determined as given above. The same experiment was repeated at pH 9.0 using 20 mM Tris-HCl. The results are summarized in Table 4. At pH 7.0 the presence of creatine reduced the radiolabel to only 14% below that of the control. All of the other effectors, including the putative transition state complex, showed comparable levels of 80% reduction in nucleotidylation. At pH 9.0 none of the additions were very effective at reducing nucleotidylation, with MgADP and MgADP−creatine being able to reduce levels by only 20 and 13%, respectively.

Increased Nucleotidylation Correlates with Decreased CK Activity. Purified CKMM (5 μg) was incubated with increasing concentrations of ATP (see Figure 2) at pH 6.8 and 37 °C as described above for determination of efficiency of modification by nucleotidylation. After the nucleotidylation reaction was completed, sample aliquots were withdrawn and residual CK activity was determined. Activity was measured by the formation of ATP by the transfer of the high-energy phosphate of phosphocreatine to ADP. ATP formation was measured by a rise in NADH absorbance at 340 nm in the presence of hexokinase and glucose-6-phosphate dehydrogenase using a modification of the method of Szaz (*13*) where the rate of change of NADH absorbance is directly proportional to CK activity. Activity measurement was initiated by the addition of creatine phosphate. The presence of ATP in the samples was compensated for by withholding creatine

Table 2: Apparent Binding Affinities and Saturation Concentrations for Various Nucleotides That Nucleotidylate CKMM and CKBB at pH 6.8 and 22 or 37 °C

	[α^{32}P]8N$_3$ATP CKBB, 22 °C	[α^{32}P]ATP CKMM, 22 °C	[α^{32}P]2N$_3$ATP CKMM, 22 °C	[α^{32}P]8N$_3$ATP CKMM, 22 °C	[α^{32}P]ATP CKMM, 37 °C
K_d (μM)	35	57	60	75	125
saturation (μM)	190	195	195	195	340

Table 3: Percent of Net ^{32}P Label (with Respect to Control) Remaining on Nucleotidylated CK after Various Time Intervals of Incubation with Creatine at pH 9.0 and 37 °C

time (min)	0	15	30	60	210
^{32}P label (%)	100	89	80	74	66

phosphate addition until all of the endogenous ATP was consumed by the hexokinase reaction. Saturation of inhibition occurred at 340 μM with a k_i of 125 μM as shown in Figure 2. Activity decreased about 12% from 394 units/L (control, no added ATP) to 343.6 units/L at approximately 340 μM ATP and remained constant through 582 μM ATP. The 12% decrease was consistent with the determined 8−10% minimal modification of the active site by nucleotidylation and suggests active site involvement.

Inhibition of Nucleotidylation by Iodoacetate. CK is known to have a very reactive sulfhydryl (C^{282}) within the ATP binding domain (*8*). To study possible involvement of this sulfhydryl in the nucleotidylation reaction CKMM was incubated with varying concentrations of iodoacetic acid (IAA), for 60 min, pH 6.8 at 37 °C. After 60 min, 250 μM [α^{32}P]ATP was added and incubation for an additional 60 min allowed to proceed. The reaction was stopped, and the level ^{32}P incorporation was determined. At 5 μM IAA, ^{32}P incorporation decreased to 16% of control and labeling was completely abolished between 40 and 80 μM IAA (data not shown).

Saturation of Auto-incorporation with Azido Nucleotide Substrates. 2N$_3$ATP and 8N$_3$ATP have previously been shown to be substrates for CK with 2N$_3$ATP having the highest apparent affinity. To determine if the azido nucleotides could serve as substitutes for [α^{32}P]ATP in the nucleotidylation reaction with CKMM, saturation studies were done at pH 6.8 and 22 °C with [γ^{32}P]8N$_3$ATP and [γ^{32}P]2N$_3$ATP in the absence of activating UV light. ^{32}P incorporation was determined, and the results obtained are shown in Figure 2 for [α^{32}P]ATP which are typical for all nucleotides tested (see also Table 2). Apparent K_d values of 60 and 75 μM were obtained for [γ^{32}P]2N$_3$ATP and [γ^{32}P]8N$_3$ATP, respectively. Therefore, the relative affinity as determined by substrate properties, photolabeling, and nucleotidylation follows the order of ATP > 2N$_3$ATP > 8N$_3$-ATP. To check for the stability of the azido analogues to the nucleotidylation conditions, [γ^{32}P]8N$_3$ATP and [γ^{32}P]2N$_3$-ATP were subjected to identical conditions only in the absence of CK. No changes in their UV absorption spectra or TLC migration were detected (data not shown). All of the azido probes, including [α^{32}P]8N$_3$ADP, were able to effectively substitute for ATP in the nucleotidylation reaction.

Removal of Nucleotidyation Radiolabel by NaIO$_4$. CK was nucleotidylated three different times using the above procedures then incubated with 100 μM NaIO$_4$ for 0−60 min to oxidize the 2′-3′ cis hydroxyls and cleave at this position. Benzylamine (200 μM) was present to react with the aldehydes produced and prevent subsequent radiolabeling of protein by Shiff's base mechanisms. Presence of benzylamine alone did not affect level of nucleotidylation. An aliquot was taken in the absence of NaIO$_4$ as the 100% level of control. In all experiments this procedure resulted in the rapid loss of all measurable radiolabel.

Inhibition of Photolabeling by Previous Nucleotidylation. Olcott et al. established that 2N$_3$ATP and 8N$_3$ATP are substrates and that photoinsertion occurs at the active site (*8*). To determine the effects of nucleotidylation on subsequent photolabeling, CK was incubated for 60 min with nonradioactive ATP to effect nucleotidylation and the unbound ATP was removed by ultrafiltration techniques (see Materials and Methods). The pre-nucleotidylated CK was then photolabeled with 50 μM [α^{32}P]8N$_3$ATP and radioincorporation measured to be 20 623 CPM (CK-nucleotidylated). Control CK treated identically except ATP was added 1 min before ultrafiltration. Radioincorporation by photolabeling was measured to be 28 506 CPM as described in Materials and Methods. In comparison to the CK−control, the nucleotidylated CK showed a 28% decrease in photolabeling.

Inhibition of Nucleotidylation by Pre-photolabeling. To determine the effect of photolabeling on subsequent nucleotidylation one aliquot of CK was first photolabeled with 100 μM nonradioactive 8N$_3$ATP, and the unbound probe was removed by ultrafiltration (see Materials and Methods). Another aliquot of the CK was treated identically except no photolysis was done. After pretreatment, both aliquots were incubated with 800 μM [α^{32}P]ATP and nucleotidylation levels were determined. The photolyzed and nonphotolyzed CK aliquots incorporated 851 and 1920 CPM, respectively. Therefore, in comparison to the CK−control, the pre-photolabeled CK showed a 56% decrease in nucleotidylation. This was consistent with the effective photolabeling of CK by 8N$_3$ATP as previously reported (*8*).

Comparison of the Tryptic Peptides from Photolabeled and Nucleotidylated CK. The following experiments were done to compare the CK peptides modified by photolabeling with [α^{32}P]8N$_3$ATP to the peptides nucleotidylated by [α^{32}P]ATP. First, identical batches of CK were photolabeled with [α^{32}P]8N$_3$ATP or nucleotidylated with [α^{32}P]ATP (or [α^{32}P]8N$_3$ATP, data not shown). These two preparations of CK were treated identically during the trypsin digestion (see Materials and Methods). The tryptic digests of both photolabeled and nucleotidylated CK were divided into halves. One-half of each preparation was subjected to reverse phase HPLC using a C8 column (see Materials and Methods) to compare the retention times of the radiolabeled peptides. The second half was subjected to tris-tricine electrophoresis to compare the electrophoretic migration patterns of the radiolabeled peptides.

Reverse Phase HPLC Analysis of Photolabeled and Nucleotidylated Tryptic Peptides from CK. For both the

Table 4: Reduction of Nucleotidylation by Substrates and Putative Transition State Complexes at pH 7.0 and 9.0 and 37 °C (^{32}P Label Is Shown as Percent of Control)

	control	creatine (Cr)	MgADP	MgADP−Cr	MgADP−Cr−NO_3
pH 7.0 (% of control)	100	86	18	20	21
pH 9.0 (% of control)	100	97	80	87	100

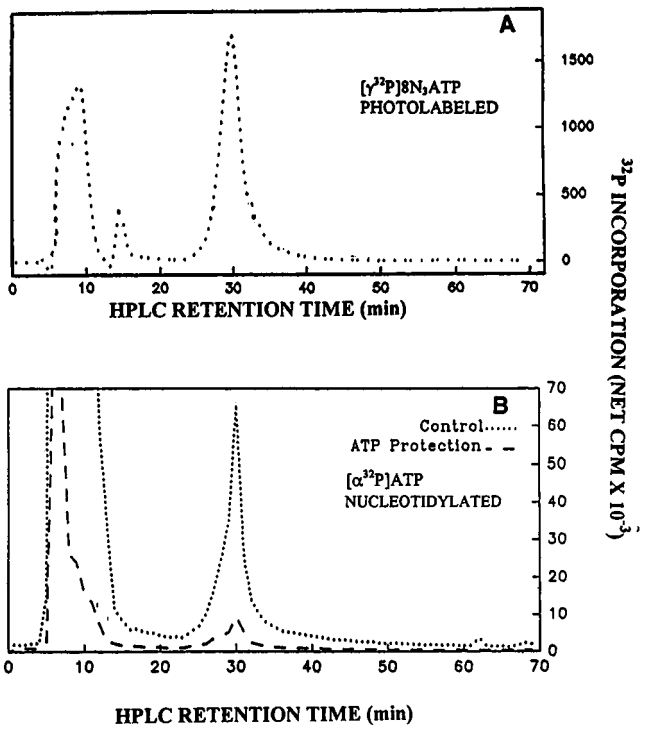

FIGURE 6: ^{32}P profile of reverse phase HPLC tryptic peptides of CK. Parameters for analysis was as described in Materials and Methods. (A) UV and ^{32}P profile of photolabeled peptide. (B) ^{32}P profile nucleotidylated tryptic digest.

photolabeled and nucleotidylated CK peptide preparations, a single radiolabeled fraction eluting at 30 min with UV absorbance was observed as shown in Figure 6. This is identical with earlier results that identified the active site peptides of CK using [γ^{32}P]2N_3ATP and [γ^{32}P]8N_3ATP and where the peptides were isolated under the same HPLC conditions (*8*). Sequencing of peptides isolated with immobilized metal ion chromatography followed by HPLC under these conditions identified these peptides as V^{279}−R^{291} (*8*). It is also consistent with the results (*7*) that demonstrated that the sequence of the peptide radiolabeled by [γ^{32}P]ATP overlapped with the sequence of the peptide identified by photolabeling. Further, the presence of ATP during either the photolabeling (*8*) or nucleotidylation procedure (see Figure 6B) greatly reduced radiolabel incorporation. The large radioactivity eluting between fractions 1−13 represent nucleotide not removed by the process before digestion. These fractions did not contain any peptide and eluted before start of the gradient (see Materials and Methods).

Combination of Reverse Phase HPLC and Tris-tricine Electrophoresis of the Tryptic Digest. The other half of the tryptic digests was subjected to Tris-tricine electrophoresis using a 16.5% T, 6% C Tris-tricine polyacrylamide gel (*14*). A single radiolabeled peptide band was observed from both preparations. Further, the radiolabeled HPLC fractions eluting at 30 min from Figure 6B, when lyophilized, resuspended in a solubilizing mixture (PSM) and subjected to Tris-tricine electrophoresis, comigrated with the radiolabeled peptides obtained when the CK tryptic digests were subjected to Tris-tricine electrophoresis alone without HPLC analysis. All of the radiolabeled fractions had the same M_r value. However, the HPLC purified fraction from Figure 6B showed an 80% loss in radiolabel in comparison to the radiolabeled peptide obtained from the tryptic digest not subjected to HPLC. This suggests that the nucleotidylated bond is unstable to these HPLC conditions.

Tris-tricine Electrophoresis of Tryptic Digest at Varying Times. This experiment was done to determine if photolabeled and nucleotidylated CK would produce the same profile of radiolabeled tryptic peptides through partial degrees of proteolysis. Equal amounts of CK were photolabeled with 100 μM [α^{32}P]8N_3ATP or nucleotidylated with 800 μM [α^{32}P]ATP. These two CK preparations were treated identically with trypsin as described above. At the given time intervals, aliquots of each digest were withdrawn and subjected to Tris-tricine electrophoresis, and radiolabeled peptides detected by autoradiography. The photolabeled and nucleotidylated [^{32}P]peptides showed identical electrophoretic patterns over time on the basis of M_r values. Both aliquots showed [^{32}P]peptides of with M_r values of approximately 13 and 9 kDa within 2 min and a final [^{32}P]-peptide of about 3 kDa appearing between 3 and 9 min (data not shown). This indicates that the radiolabel from both nucleotidylation and photolabeling is in the same peptide region. The M_r values of small peptides with three additional phosphates attached are not very accurate and always migrate as larger species. This is evidenced by the HPLC-isolated peptide (approximately 1480 kDa by sequence analysis) also having an M_r of about 3 kDa on the same gel.

Reduction of [α ^{32}P]ATP Nucleotidylation into CK Tryptic Peptide by ATP. One milligram of CK was incubated with 2.5 mM ATP at pH 8.5 (Tris-HCl), 37 °C to effect maximum nucleotidylation. An additional identical preparation of CK was done without added ATP. Both batches were treated by ultrafiltration to remove any ATP present. [α^{32}P]ATP (225 μM) was added, and the batches were incubated for an additional 60 min to effect nucleotidylation. The reactions were stopped by TCA precipitation and centrifuged, and the pellet was digested with trypsin as above. The digest was then subjected to reverse phase HPLC on a C8 column as described in Materials and Methods. The peptide from the CK pretreated with ATP showed an 86% reduction of nucleotidylation by [α^{32}P]ATP (see Figure 6B). This eliminated the possibility that the [α^{32}P]ATP contains a nonspecific protein reactive species generated during the exchange reaction that is responsible for the nucleotidylation.

DISCUSSION

When control human brain homogenates were incubated with [α^{32}P]ATP or [α^{32}P]ADP and subjected to SDS-PAGE, the association of radioactivity with CK indicated the

presence of a covalent linkage between at least part of the nucleotide and CK that was obviously not a γ-phosphorylation. This was a previously unreported observation. As shown in Figure 1, of the many ATP binding enzymes present in human brain, we found only five or six enzymes that significantly auto-incorporate radiolabel from [α^{32}P]ATP. Also, of the five to six proteins which auto-incorporated radiolabel from [α^{32}P]ATP, only CK in Alzheimer's disease brain showed abolished radiolabeling giving medical relevance to this observation (*1*). It is a possibility that the inhibition of CK by nucleotidylation is what causes the reduction of CK activity and photolabeling in AD brain.

This work also demonstrates that purified CK cytosolic isozymes auto-incorporate the entire ATP molecule in a nucleotidylation process. The occurrence of this nucleotidylation with purified CK, as well as with CK in brain homogenates, reduces the possibility of a second enzyme being involved in this modification. Further, it was demonstrated by several techniques that nucleotidylation occurred within the ATP binding site of CK and resulted in the inhibition of CK activity. These observations also correct a prior assumption that auto-incorporation of radiolabel by [γ^{32}P]ATP into CK was due to an autophosphorylation process.

Chicken and rat CKBB and rabbit CKMM (*3*−*6*) have been proposed to be posttranslationally modified by phosphorylation. In light of the data presented here, it is likely that nucleotidylation, rather than autophosphorylation, was the posttranslational modification occurring. Since nucleotidylation occurred at the active site of CK, it may have a role to play in regulation although this remains unexplained. It may represent an inherent weakness in CK stability to oxidation or heavy metal toxicity and, as such, may be connected with the aberrant behavior of CK observed in Alzheimer's disease.

The mechanism of CK auto-incorporation or nucleotidylation would be of obvious importance. Phosphorylation and adenylylation were ruled out as [α^{32}P]ATP and [γ^{32}P]ATP auto-incorporate label into CK equally within a 5% experimental error. Also, at pH 6.8, [α^{32}P]ADP auto-incorporated with a 28% greater efficiency than [α^{32}P]ATP. The incorporation of [γ^{32}P]ATP and [2,8-^{3}H]ATP strongly supported the concept that the entire ATP molecule was being incorporated in a nucleotidylation process.

To confirm that the observed incorporation of [α^{32}P]ATP into CK was not an artifact and was specific for CK, various other ATP binding enzymes, including other kinases, were incubated with [α^{32}P]ATP. None of these enzymes, other than CK, incorporated significant amounts of [α^{32}P]ATP. This eliminated the possibility that attachment of ATP to CK is through any oxidized species of ATP or some other generally reactive chemistry. Only a maximum of 8−10% of all copies of CK were nucleotidylated, even at very high ATP concentrations. Therefore, if chemically reactive species do exist, they are within the enzyme.

Selective protection against nucleotidylation by the native substrates ATP, ADP, and phosphocreatine (at 22 °C) was observed. Also, data was obtained that demonstrated the inability of structurally similar compounds such as AMP, GTP, GMP, NAD, and creatine to elicit a significant level of reduction of nucleotidylation by [α^{32}P]ATP and [α^{32}P]-ADP (see Table 1). CK is known to have a higher affinity for ADP at pH 7.0 than at pH 9.0 (*15*). In agreement with this, at 37 °C, ADP inhibited [γ^{32}P]ATP nucleotidylation by only 20% at pH 9.0 but by 93% at pH 7.0 in response to the tighter binding of ADP at pH 7.0 (see Table 4). This mimicking of the known nucleotide binding pattern of CK strongly supported the concept that nucleotidylation occurred specifically at the ATP binding site.

While creatine and ATP have distinct binding domains, the phosphate of phosphocreatine and the γ-phosphate of ATP would likely have overlapping regions in the CK active site. In agreement with this, creatine did not significantly inhibit [γ^{32}P]ATP nucleotidylation into CK at either pH 7.0 or 9.0 (Table 4). However, phosphocreatine significantly reduced ATP nucleotidylation (Table 1). This also implies that nucleotidylation occurred on the basis of binding to the ATP/ADP binding domain rather than at the creatine binding domain.

A previous study had shown that the azido analogues of ATP, [γ^{32} P]8N_3ATP, and [γ^{32} P]2N_3ATP effectively mimic ATP as a substrate in the CK reaction. These probes were used to photolabel and identify two peptides within the adenine binding domain of the ATP binding site (*8*). The crystallization of ATP−mitochondrial CK confirmed the location of these peptides as being near the adenine ring and gave detailed orientation of ATP within the CK structure (*9*). Olcott et al. showed that the efficiency for the production of phosphocreatine was in the order of [γ^{32}P]ATP > [γ^{32}P]2N_3ATP > [γ^{32}P]8N_3ATP. In the absence of activating UV light, these probes effectively nucleotidylate CK (Figure 5 and Table 2). Also, the nucleotidylation with azido probes saturated giving apparent binding affinities in the same order as observed for phosphocreatine synthesis. This order of affinities was to be expected since ATP and 2N_3ATP are primarily in the native anti conformation and thus bind to CK with tighter affinity than 8N_3ATP which is primarily in the syn conformation.

Further support for active site interaction was indicated by the kinetics of nucleotidylation of the different isozymes of CK. With [α^{32}P]8N_3ATP, CKBB gave a $k_{0.5}$ value of 35 μM while CKMM had a $k_{0.5}$ value of 60 μM at pH 6.8 and 22 °C. The tighter affinity of CKBB over CKMM for nucleotidylation was supported by the report of a K_m of 110 μM for CKBB and 220 μM for CKMM under slightly different conditions at pH 6.8 (*16*). Also, Witteveen and Morin reported a similar substrate affinity between CKBB and CKMM (*17*, *18*). These results showed that the increased efficiency related to nucleotidylation was correlated with ATP and analogue binding properties.

Experiments confirmed that photolabeling reduced nucleotidylation and vice versa. Photolabeling CK first with cold 8N_3ATP should modify a percentage of the active sites of the CK and decrease subsequent nucleotidylation with [α^{32}P]ATP. As expected, 8N_3ATP-photolabeled CK showed a 56% decease in nucleotidylation with [α^{32}P]ATP. Also, CK nucleotidylated and subsequently photolabeled showed a 28% decrease in photoinsertion of [α^{32}P]8N_3ATP relative to only photolabeled CK (control). These results are supportive of both of the processes occurring within the ATP binding site.

Studies on nucleotidylation with [α^{32}P]ATP showed a $k_{0.5}$ of 125 μM and saturation of ^{32}P incorporation at 340 μM. Inhibition of CK activity was 50% of maximum inhibition

when nucleotidylation was effected at 125 μM ATP and maximum inhibition of activity occurred at 340 μM ATP (see Figure 2). Maximum nucleotidylation modified 8 to 9% of CK and inhibition of CK activity was 14%. Thus, using these two independent techniques to monitor fractional active site modification of CK by nucleotidylation yielded very similar results. The data agreed with the previously reported value of less than 10% efficiency of CK radiolabeling with [γ^{32}P]ATP reported as a phosphorylation (*7*).

From [α^{32}P]ATP nucleotidylated CK a single tryptic [^{32}P]-labeled peptide was obtained after immobilized column chromatography which comigrated on reverse phase HPLC with [^{32}P]-labeled peptide isolated from CK after photolabeling with [α^{32}P]8N_3ATP (Figure 6). Further, tris-tricine electrophoresis of radiolabeled peptides produced by timed trypsin proteolysis produced the same M_r profile of 13, 9, and 3 kDa [^{32}P]peptides using nucleotidylated or photolabeled CK. Both of the nucleotidylated and photolabeled tryptic peptides purified by HPLC comigrated on tris-tricine gels with an apparent M_r value near 3000 kDa. This peptide ($V^{279}-R^{291}$) had previously been identified as a radiolabeled peptide modified by photoaffinity labeling (*8*). Also, as expected, the incorporation of the triphosphate slowed the migration rate giving larger M_r values. This tryptic peptide has an overlap with the 4500 kDa endoproteinase Lys-C of the putative phosphopeptide produced by [^{32}P]ATP (*7*). Also, preincubation with ATP reduced by 86% the [^{32}P]-labeled peptide isolated by reverse phase HPLC after [α^{32}P]ATP nucleotidylation (see Figure 6B). Therefore, photolabeling and nucleotidylation appeared to be modifying the same peptide. Identification of the residue involved in nucleotidylation by sequencing was made difficult by the presence of threonine and serine within two residues of C^{282}. Cysteine is not detected by our sequencing procedure, and T and S are usually detected in lower yields than other amino acids (*8*).

In an attempt to elucidate the mechanistic aspects of this phenomena, we studied the effects of creatine, phosphocreatine, nucleotide substrates and putative transition state complexes on the nucleotidylation reaction. If nucleotidylated CK was a covalent intermediate formed as part of the transphosphorylation reaction, then the addition of creatine to the nucleotidylated CK species should lead to the rapid loss of the [γ-^{32}P]phosphate and abolish CK label. However, it took 30 min to decrease label by only 20% relative to control (Table 4). This strongly indicated that the covalent species that was formed when CK was nucleotidylated with ATP was not an intermediate in the CK transphosphorylation reaction. This result was expected on the basis of prior studies on the mechanism of CK (*19*).

The transphosphorylation reaction has been proposed to involve a direct in-line transfer of the γ-phosphate from ATP to creatine. This is an SN2 type reaction, with the phosphoryl group forming a planar sp^3d hybrid in the transition state (*20*). Pauling and Jencks pointed out that a molecule resembling its transition state in the conversion from substrate into product might be expected to bind more tightly to the enzyme than the substrate molecules alone (*21*, *22*). The NO_3^- ion, in combination with creatine and MgADP, has been reported to mimic this transition state complex. For example, it has been shown that the binding of both creatine and MgADP, as components of the enzyme−creatine−MgADP anion complex, was stronger than their apparent binding as individual substrates (*20*). However, the putative transition state complex, Mg−ADP−creatine−nitrate, while it did reduce nucleotidylation, did not do much better than either MgADP or MgADP−creatine. The same had been observed for photolableing the active site with [α^{32}P]8N_3-ATP (data not shown). These results implied that the Mg−ADP−creatine−NO_3 complex neither resembles the transition state of the nucleotidylation reaction nor binds to the active site significantly tighter than MgADP alone.

To understand the nature of this unique attachment of ATP with CK, various nucleotide derivatives were used in our studies. [2,8-^{3}H]ATP could also be used to radiolabeled CK showing that the base was retained. The 2 and 8 positions of adenine are blocked by the azido group in [α^{32}P]2N_3ATP and [α^{32}P]8N_3ATP, both of which effectively nucleotidylated into CK. Nucleotidylation of CK by [γ^{32}P]TNP−ATP, which has the 2′ and 3′ sugar hydroxyls blocked, proved these positions did not participate in the nucleotidylation reaction and decreased the possibility of an Amadori rearrangement being involved. [γ^{32}P]ATPγBP is modified on the radiolabeled γ-phosphate and represents a class of non-hydrolyzable ATP analogues. Effective nucleotidylation with [γ^{32}P]-ATPγBP, as well as effective nucleotidylation by [α^{32}P]ADP, proved that phosphorylation was not occurring and that nucleotidylation did not require the γ-phosphate. This left the 1′ carbon of the ribose as the most likely position at which attachment of the ATP molecule was occurring. The observation that $NaIO_4$ cleavage of the 2′-3′ bond results in the total loss of radiolabel attached through nucleotidylation implied that attachment was through the 1′ carbon which obligated opening of the ribose ring. This ring opening would cause release of the [^{32}P]phosphates upon oxidation of the cis hydroxyls as was observed.

CK has a single very reactive cysteine residue (Cys^{282}) within the active site that is readily modified by several sulfhydryl reagents, leading to decreased levels of enzyme activity including total inhibition (*23*−*25*). Iodoacetamide and epoxycreatine, respectively, have been shown to bind at Cys^{282} and inactivate CK (*26*, *27*). The ability of 40−80 μM iodoacetic acid to abolish nucleotidylation of CK [α^{32}P]-ATP supported the concept that nucleotidylation is occurring within the active site of CK and likely involved C^{282}. Therefore, it is most likely that the attack occurred on the 1′carbon in the nucleotidylation process and most likely is through the thiol group of C^{282}.

The fact that the temperature-dependent increase in nucleotidylation was much greater with CK in brain homogenates versus purified CK (a 5-fold increase versus a 77% increase going from 4 to 22 °C) indicated that the presence of certain chemicals in the homogenate enhanced nucleotidylation. It is quite possible that the observed nucleotidylation of CK is dependent on an undesirable reactivity of this enzyme with other cellular components at the active site that leads to a reactive species. This observation may be important in explaining the mechanism of CK inhibition in Alzheimer's disease and is currently under investigation.

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