Autophosphorylation of the Mammalian Multifunctional Protein That Initiates de Novo Pyrimidine Biosynthesis*

Frederic D. Sigoillot, David R. Evans, and Hedeel I. Guy‡

From the Department of Biochemistry and Molecular Biology, Wayne State University School of Medicine, Detroit, Michigan 48201

CAD, a large multifunctional protein that carries carbamoyl phosphate synthetase (CPSase), aspartate transcarbamoylase, and dihydroorotase activities, catalyzes the first three steps of de novo pyrimidine biosynthesis in mammalian cells. The CPSase component, which catalyzes the initial, rate-limiting step, exhibits complex regulatory mechanisms involving allosteric effectors and phosphorylation that control the flux of metabolites through the pathway. Incubation of CAD with ATP in the absence of exogenous kinases resulted in the incorporation of 1 mol of P_i/mol of CAD monomer. Mass spectrometry analysis of tryptic digests showed that Thr1037 located within the CAD CPS.B subdomain was specifically modified. The reaction is specific for MgATP, ADP was a competitive inhibitor, and the native tertiary structure of the protein was required. Phosphorylation occurred after denaturation, further purification of CAD by SDS gel electrophoresis, and renaturation on a nitrocellulose membrane, strongly suggesting that phosphate incorporation resulted from an intrinsic kinase activity and was not the result of contaminating kinases. Chemical modification with the ATP analog, 5'-p-fluorosulfonylbenzoyladenosine, showed that one or both of the active sites that catalyze the ATP-dependent partial reactions are also involved in autophosphorylation. The rate of phosphorylation was dependent on the concentration of CAD, indicating that the reaction was, at least in part, intermolecular. Autophosphorylation resulted in a 2-fold increase in CPSase activity, an increased sensitivity to the feedback inhibitor UTP, and decreased allosteric activation by 5-phosphoribosyl-1-pyrophosphate, functional changes that were distinctly different from those resulting from phosphorylation by either the protein kinase A or mitogen-activated protein kinase cascades.

CAD (1–3) is a multifunctional protein that catalyzes the first three steps of the de novo pyrimidine biosynthetic pathway in mammalian cells. The protein consists of multiple copies of a 243-kDa polypeptide organized into discrete domains (Fig. 1A) that have glutamine-dependent carbamoyl phosphate synthetase (CPSase),¹ aspartate transcarbamoylase (ATCase) and dihydroorotase (DHOase) activities.

The CPSase activity, the first committed and rate-limiting step in the pathway, is the locus of control. Most CPSases share a common catalytic mechanism (4) in which the synthesis of carbamoyl phosphate proceeds through a complex series of partial reactions catalyzed by different functional domains.

Glutamine + H₂O → glutamate + NH₃
HCO₃⁻ + ATP → carboxylyl + ADP
Carboxylate + NH₄⁺ → carbamate + Pᵢ
Carbamate + ATP → carbamoyl phosphate + ADP

REACTIONS 1–4

CPSases also have homologous domain structures. Glutamine hydrolysis (Reaction 1) occurs on the 40-kDa glutaminase domain or subunit. The synthetase (CPS) domain or subunit consists of two homologous 60-kDa subdomains, CPS.A and CPS.B. The activation of bicarbonate and the subsequent reaction with NH₃ (Reactions 2 and 3) occurs on CPS.A, whereas the phosphorylation of carbamate to form carbamoyl phosphate (Reaction 4) occurs on CPS.B (5). Lusty recognized that the CPS domain (6) evolved by gene duplication, translocation, and fusion of an ancestral kinase gene.

CAD CPSase also regulates the flux of metabolites through the de novo pyrimidine pathway (7) via a complex network of interacting control mechanisms. The CPS domain is subject to allosteric activation by 5-phosphoribosyl-1-pyrophosphate (PRPP) and feedback inhibition by UTP (8–14). Domain swapping experiments (15, 16) showed that both allosteric ligands bind to the extreme carboxyl end of CPS.B (Fig. 1A), the B3 regulatory subdomain.

Carrey (14, 17, 18) discovered that purified CAD is phosphorylated by cAMP-dependent protein kinase A (PKA). Phosphorylation does not alter the catalytic activity of CPSase or any of the other CAD activities but results in the loss of sensitivity to the allosteric inhibitor, UTP. There are two PRP phosphorylation sites, one located within the B3 regulatory subdomain (Ser1805) and a second (Ser1906) in the interdomain linker that connects the ATC and DHO domains (Fig. 1A). Desensitization of CAD to UTP results from phosphorylation of Ser1406 in the regulatory subdomain (17, 19, 20). Sahay et al. (16) subsequently discovered that the response to PRPP is also diminished by PKA phosphorylation.

* This work was supported by National Institutes of Health Grants GM/CA60371 and CA60371. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Dept. of Biochemistry and Molecular Biology, Wayne State University School of Medicine, 540 E. Canfield Ave., Detroit, MI 48201. Tel.: 313-577-1502; Fax: 313-577-2765; E-mail: hguy@ cmb.biosci.wayne.edu.

¹ The abbreviations used are: CPSase, carbamoyl phosphate synthetase; ATCase, aspartate transcarbamoylase; DHOase, dihydroorotase; FSBA, 5'-fluorosulfonylbenzoyladenosine; MAPK, mitogen-activated protein kinase; PKA, cAMP-dependent protein kinase A; Ser(P), phosphoserine; Thr(P), phosphothreonine; PRPP, 5-phosphoribosyl-1-pyrophosphate; AMPPNP, 5'-adenyl-γ-imidodiphosphate.
Autophosphorylation of a Pyrimidine Biosynthetic Complex

Fig. 1. CAD domain structure and regulatory sites. A, the 243-kDa CAD polypeptide is organized into domains that have glutamine-dependent carbamoyl phosphate synthetase activity (glutaminase [GLN], CPS.A, and CPS.B domains), aspartate transcarbamoylase activity (ATC domain), and dihydroorotase activity (DHO domain). The CPS.A and CPS.B domains are each comprised of three subdomains (A1, A2, and A3 and B1, B2, and B3). The B3 subdomain binds the allosteric ligands, UTP and PRPP, and contains the PKA phosphorylation site (PKA2) in the interdomain linker connecting the DHO and ATC domains. MAPK phosphorylates a threonine residing in the A1 subdomain. The site of autophosphorylation is located within the B1 subdomain close to the B1-B2 junction. B, the sequence surrounding the autophosphorylation site (Thr1037) of hamster CAD (Hams II, accession number P08955) is shown aligned with the corresponding region of the human (Human II, accession number XP_002428), shark (Shark II, accession number Q91437), dogfish (Dogfish II, accession number A57541) and Saccharomyces cerevisiae (Yeast II, accession number QZBYU2) pyrimidine-specific CPSases. The mitochondrial, ammonia-dependent CPSase I from human (Human I, accession number XP_040883), rat (Rat I, accession number P07756), and Xenopus (Xeno I, accession number P31327) as well as CPSase III from shark (Shark III, accession number AAA84355) and E. coli CPSase (Eco, accession number P09686) are also included in the alignment. The autophosphorylation site, Thr1037 in CAD, is boxed.

CAD was also found (21) to be regulated both in vivo and in vitro by the MAPK cascade. MAPKs (22) are ubiquitous components of the mitogen activated cascade that is involved in cellular proliferation in response to growth factors and have also been shown to be activated by oncogene products (23). MAPK phosphorylates Thr1037 in the A1 subdomain of the CAD CPS (Fig. 1A). MAPK-mediated phosphorylation, like that of PKA, abolishes UTP inhibition; however, PRPP activation is markedly stimulated. Both the loss of sensitivity to UTP and increased sensitivity to PRPP would be expected to activate CPSase and thus is likely to be important for regulation of pyrimidine biosynthesis.

We have recently found (24) that the growth-dependent activation and down-regulation of pyrimidine biosynthesis is a consequence of the sequential action of MAPK and PKA. The CPSase activity of CAD is up-regulated by the MAPK cascade with stimulators enter the proliferative phase and is subsequently down-regulated in resting cells by PKA-mediated phosphorylation.

During her seminal studies of PKA phosphorylation of CAD, Carrey noticed (17) that radiolabeled phosphate was incorporated into CAD when it was incubated with [γ-32P]ATP in the absence of exogenous kinases, a phenomenon that was attributed to either autophosphorylation or the presence of contaminating kinases. Among the estimated 1000 protein kinases in mammalian cells (25), intra- or intermolecular autophosphorylation has been found to be a common theme. Moreover, there are many examples, including cyclic nucleotide-dependent protein kinases, calcium/calmodulin-dependent protein kinases and protein kinase C (26), where autophosphorylation is known to play an important role in the regulation of the kinase. While autophosphorylation is a common, perhaps universal characteristic of protein kinases (26), it is less frequently observed in kinases that phosphorylate small molecules. Autophosphorylation of yeast hexokinase PII (27, 28) results in the inactivation of the enzyme. Creatine kinase was initially reported to be autophosphorylated (29), but this observation was subsequently shown (30) to be due to the incorporation of the entire nucleotide into the active site of the enzyme.

Here we show that incubation of CAD with ATP resulted in incorporation of the γ-phosphate of the nucleotide into a specific threonine residue in CPS.B, that the reaction was catalyzed by CAD itself and was not the result of contaminating kinases, and that autophosphorylation resulted in increased CPSase activity and a modulation of the effects of the allosteric ligands, UTP and PRPP.

EXPERIMENTAL PROCEDURES

CAD Purification—CAD was isolated from BHK 165-23 (31), a baby hamster kidney cell line derived from BHK-21 in which the CAD gene was amplified by exposure to the ATCase inhibitor, N-phosphonacetyl-l-aspartate (NPA). The protein was purified to homogeneity by 40% ammonium sulfate precipitation followed by gel filtration chromatography on a Bio-Gel A-5m column (Bio-Rad) as described previously (3). In some instances, CAD was further purified by affinity chromatography on an N-phosphonacetyl-l-aspartate column (32). CAD was also isolated from Escherichia coli transformed with pCKCAD10, a plasmid expressing the full-length CAD polypeptide as described previously (33). Escherichia coli CPSase was purified from E. coli pLLK12 transformants following the protocol described by Guillou et al. (34). Protein quantitation was performed by the Lowry method (35) and by scanning stained SDS-polyacrylamide gels calibrated with known amounts of bovine serum albumin using the software UNSCAN-IT (Silk Scientific Corp.).

Antibodies—The phosphoserine (Z-PS1) and phosphothreonine (Z-PT1) rabbit polyclonal antibodies from Zymed Laboratories Inc. were used for immunoblotting at a dilution of 1:1000 to 1:2000. The rabbit polyclonal CAD serum prepared as previously described (36) was used at a 1:5000 dilution.

Phosphorylation Reactions—CAD at a concentration of 20–200 μg/ml in a kinase buffer consisting of 50 mM Tris-HCl, pH 7.5, 10 mM MgCl2, 0.48 mg/ml bovine serum albumin was reacted at 30 °C with ATP at the concentrations indicated. The extent of phosphorylation was determined by immunoblotting or by measuring the incorporation of 32P-inorganic phosphate.

Immunoblotting—SDS-polyacrylamide gel electrophoresis on 5 or 10% gels was carried out as described by Laemmli (37). The samples were heated at 100 °C for 4 min in loading buffer prior to electrophoresis. The proteins were separated and transferred onto a 0.45-μm nitrocellulose membrane (Bio-Rad) as described by the manufacturer. The analysis was performed using the ECL reagents (Amersham Biosciences) as described previously (24). Signals were visualized using Biomax ML film (Eastman Kodak Co.) and quantitated by scanning the immunoblots with a HP ScanJet 4c and the software UNSCAN-IT (Silk Scientific). Care was taken to ensure that all exposures fell within the linear response range of the film.

CAD Enzyme Assays—The glutamine-dependent CPSase assay was carried out as previously described (33). The assay mixture (1 ml) contained 20–60 μg of protein, 100 mM Tris-HCl, pH 8.0, 100 mM KCl, 7.5% MeSO4, 2% glycerol, 1 mM dithiothreitol, 3.5 mM glutamine, 20 mM aspartate, 1.5 mM ATP, 3.5 mM MgCl2, and 5 mM sodium [14C]-bicarbonate (1.6 × 106 μCi/ml). When UTP or PRPP were included, the concentration of MgCl2 was adjusted in order to maintain a 2 mM excess over the sum of the concentrations of ATP, UTP, and PRPP. The reaction was performed at 37 °C for 15 min and quenched by the addition of 1 ml of 40% trichloroacetic acid and heated at 100 °C for 15 min. Approximately 0.2 g of dry ice was added to the vials to eliminate
the excess CO₂ generated during the reaction. The sample was then heated at 100 °C for an additional 15 min prior to counting in a Beckman-Coulter counter.

ATCase and DHOase Assays—The ATCase and DHOase activities were determined using the previously described colorimetric method (38, 39). The ATCase assay mixture contained 5 mM carbamoyl phosphate and 12 mM aspartate in a buffer consisting of 100 mM Tris-HCl, pH 8.0, 100 mM KCl, 7.5 mM MgSO₄, 2.5% glycerol, and 1 mM diithiothreitol. The DHOase activity was assayed by monitoring the conversion of 1 mM dihydroorotate to carbamoyl aspartate in 25 mM HEPES, pH 7.5, 5% glycerol.

On-membrane Kinase Assay—The on-membrane kinase assay was carried out according to Ferrell et al. (40). Briefly, purified CAD (30–65 μg) was separated on a 5% SDS-polyacrylamide gel and transferred onto a nitrocellulose membrane. The protein was then incubated for 1 h at room temperature with gentle rocking in a buffer containing 7 M guanidine hydrochloride, 50 mM Tris-HCl, pH 8.3, 50 mM diithiothreitol, and 2 mM EDTA. The protein was renatured on the membrane for 16–18 h at 4 °C by incubation in a buffer containing 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 2 mM diithiothreitol, 2 mM EDTA, 1% (v/v) bovine serum albumin (Sigma), and 0.1% Igepal CA-630 (Sigma). The nitrocellulose membrane was then washed with a buffer containing 5% (v/v) bovine serum albumin, as a blocking agent, in 30 mM Tris-HCl, pH 7.5, at room temperature for 1 h. The membrane was then incubated with 150 μCi of [γ-32P]ATP (ICN) (4500 mCi/mmol) in 10 ml of kinase buffer consisting of 30 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 2 mM MnCl₂, for 5 h. The reaction was quenched by washing the membrane twice in 30 mM Tris-HCl, pH 7.5. The membrane was further washed once with a buffer containing 30 mM Tris-HCl, pH 7.5, 0.05% Igepal CA-630 and once in a buffer containing 30 mM Tris-HCl, pH 7.5, for 10 min each at room temperature. The membrane was then washed 10 min at room temperature in 1 M KOH and rinsed several times in 10% (v/v) acetic acid. The membrane was then washed on a Storage phosphor screen, and the signals were captured with the Packard Cyclone Storage phosphor system. The membrane was also incubated after the blocking step above with 0.5 mM cold ATP in the kinase buffer and then subjected to Thr(P) immunoblotting and quantitation as described above.

Mass Spectrometry Sequence Analysis—The phosphorylated residues were identified by liquid chromatography-mass spectrometry. CAD (330 μg) was subjected to SDS-polyacrylamide gel electrophoresis. After staining and destaining, the CAD bands were excised from the gel and pooled. The gel slices were washed twice with 50% acetonitrile in water and then frozen at −20 °C. The protein was digested in situ with trypsin, and sequence analysis was performed at the Harvard Microchemistry Facility by microcapillary reverse-phase high pressure liquid chromatography nanoelectrospray tandem mass spectrometry (LC/MS/MS) on a Finnigan LCQ DECA quadrupole ion trap mass spectrometer.

Stoichiometry of CAD Phosphorylation—The stoichiometry of CAD autophosphorylation was determined by the method described by Carrey et al. (17). Purified CAD (21.3 μg) was incubated in kinase buffer with 200 μCi [γ-32P]ATP (200 μCi/μmol) in a final volume of 100 μl for 120 min. Bovine serum albumin (20 μg) was added as a carrier, and the protein was precipitated by the addition of 50 μl of 25% trichloroacetic acid. After 30 min on ice, the sample was centrifuged at 24,000 × g at 4 °C for 30 min. The trichloroacetic acid precipitate was washed twice with 25% trichloroacetic acid and resuspended in 1 mM NaOH. Scintillation mixture (RPI) was added (10 μl), and the sample was counted in a Beckman Coulter LS 6500 scintillation counter.

RESULTS

Phosphorylation of CAD—While investigating the phosphorylation of purified CAD by PKA and MAPK, we noticed a persistent background phosphorylation. To further characterize this phenomenon, CAD was incubated with 0.5 mM ATP, and samples were withdrawn at the indicated times (Fig. 2). Analysis by immunoblotting using antibodies directed against Thr(P) gave a strong signal and showed that the phosphorylation occurred slowly over a period of 2 h (Fig. 2A). No phosphorylation could be detected with phosphoserine (Ser(P)) antibodies (Fig. 2B, lanes 1–8), although control samples (lanes 9 and 10) using CAD phosphorylated with PKA indicated that the antibodies reacted strongly and specifically with the serine residues known to be phosphorylated by this kinase. The intensity of the signals was quantitated by scanning the immunoblots. The progress curve for Thr phosphorylation (Fig. 2C) was distinctly sigmoidal and began to level off after about 1 h. A time course extended over a period of 7 h (not shown) indicated that no additional phosphorylation occurred after 2 h.

Quantitation of the extent of phosphorylation resulting from six different phosphorylation experiments, determined by measuring the incorporation of either [γ-32P]ATP or [γ-33P]ATP into CAD during a 2-h reaction period (see “Experimental Procedures”), revealed that there was 0.97 ± 0.16 mol of phosphate incorporated/mol of CAD monomer.

Criteria for Phosphorylation—A series of reactions (Fig. 3) were carried out to assess the requirements for phosphorylation. In contrast to the extensive threonine phosphorylation observed following incubation of CAD with 0.5 mM ATP (Fig. 3A, lane 3), immunoblotting of unreacted CAD (lane 1) or of CAD incubated in the absence of ATP (lane 2) revealed only very low levels of threonine and serine phosphorylation. In the presence of the serine/threonine-specific protein phosphatase 1, the extent of phosphorylation was markedly reduced (lane 4). No phosphorylation occurred when CAD was heat-denatured (lane 5) prior to incubation with ATP or if the reaction was
Phosphorylation of CAD proceeded in the presence of MgATP and other CPSase substrates, bicarbonate and glutamine (lane 7). Thr(P) phosphorylation also occurred when PKA was present and disrupt any putative CAD-kinase complexes. CAD was then renatured on the nitrocellulose membrane and incubated with [$\gamma$-32P]ATP. Autoradiography clearly showed that CAD is phosphorylated and that the extent of phosphorylation increased linearly (Fig. 4) with the amount of CAD applied to the SDS gel. Alternatively, the membrane was incubated with unlabeled ATP and then probed with Thr(P) and Ser(P) antibodies (not shown). This approach confirmed that phosphate incorporation was incorporated into threonine residues.

CAD isolated from E. coli cells transformed with pCKCAD10 (33), a plasmid expressing the full-length CAD polypeptide, was also found to be phosphorylated to the same extent as the CAD isolated from mammalian cells (data not shown). The relative paucity of Ser/Thr kinases in bacterial cells also argues against the involvement of contaminating kinases in the CAD phosphorylation. In contrast, purified E. coli CPSase exhibited no phosphorylation despite the conservation of the target threonine residue described below (Fig. 1B).

Collectively, these experiments provide strong support for an intrinsic CAD kinase activity and suggest that phosphate incorporation is the result of an autophosphorylation reaction.

**Liquid Chromatography-Mass Spectrometry Analysis of Autophosphorylated CAD**—To determine the site of autophosphorylation, CAD (0.3 mg) was reacted for 6 h with 0.5 mM ATP and subjected to SDS-polyacrylamide gel electrophoresis. The protein bands were excised, exhaustively digested with trypsin, and analyzed by liquid chromatography-mass spectrometry at the Harvard Microchemistry Facility. The analysis clearly revealed two major phosphopeptides (Table I). Phosphopeptide 1 was located in the CPS.B B1 subdomain close to the junction with the B2 subdomain (Fig. 1A). The sequence of this peptide revealed tandem threonine and serine residues. The analysis favored phosphoserine, although phosphorylation of the adjacent threonine residue could not be ruled out. Phosphopeptide 2 corresponded to the serine residue located in the interdomain linker connecting the DHO and ATC domains shown previously (17) to be phosphorylated by PKA. There was an indication of a third phosphopeptide located within the glutaminase domain.
Autophosphorylation sites are underlined.

### Table I

| Peptide | Target residue | Domain         | CAD residue nos. | Sequence             |
|---------|----------------|----------------|------------------|----------------------|
| 1       | Thr/Ser        | CPS.B1         | 1034–1048        | VALTSPKIAEDSAENR     |
| 2       | Ser            | DA linker      | 1855–1869        | IVRA5DGPEAEEDK       |
| 3       | Ser            | GLN            | 138–168          | LVQSEQTEPESTLPFNARPLAPEVISIKTPR |

*Low confidence assignment but possible phosphopeptide.*

**Kinetics of CAD Autophosphorylation**—CAD was incubated at 30 °C for 30 min with various ATP concentrations, and the extent of autophosphorylation was determined by scanning immunoblots probed with Thr(P) antibodies (Fig. 5). The resulting ATP saturation curve was hyperbolic, although significant substrate inhibition was apparent at ATP concentrations exceeding 2 mM. The ATP saturation curve of CPS.B exhibits similar inhibition at high ATP concentrations (41). The $K_m$ for ATP for the CAD autophosphorylation reaction was 0.33 ± 0.026 mM.

The rate of autophosphorylation was determined using 0.085 μg/μl CAD and a 3-fold dilution of the protein, 0.028 μg/μl. The time course (Fig. 6A) showed that the protein did not significantly reduce the rate of phosphorylation, although the maximum extent of phosphate incorporation, expressed per μg of CAD, was approximately the same. In a second experiment, various concentrations of CAD were incubated with 0.5 mM ATP for 1 h. The plot of phosphorylation rate versus CAD concentration (Fig. 6B) is not linear as expected for a first order intramolecular reaction (dashed and dotted line), nor does it conform to simple second order kinetics (dashed line) expected if autophosphorylation were a strictly intermolecular process. While the interpretation of this result is difficult, since CAD serves as both substrate and enzyme, it is likely that both cis- and trans-autophosphorylation occur.

**The Effect of Allosteric Ligands on Autophosphorylation**—Since the autophosphorylation reaction is catalyzed by the CPSase active sites, the phosphorylation rate was determined in the presence of allosteric effectors that modulate the CPSase activity of the complex. UTP was found (Fig. 7) to strongly inhibit autophosphorylation. As the concentration of UTP increased, the rate of phosphorylation decreased, leveling off at about 20% (Fig. 7C) of the rate in the absence of the nucleotide. The observation that higher UTP concentrations did not result in further rate reduction indicates that UTP was not acting as a competitive inhibitor of ATP but rather allosterically inhibited autophosphorylation. The conformational changes induced by the binding of UTP to the regulatory domain primarily reduce the catalytic activity of CPS.B (44), but also indirectly inactivate CPS.A.

PRPP increased the rate of autophosphorylation at concentrations up to about 200 μM. The decrease in rate seen at higher, unphysiological PRPP concentrations was not the result of competitive inhibition, since there was no corresponding reduction in CPSase activity at these concentrations of the activator (data not shown).

**Effect of Autophosphorylation on the Catalytic Activity and Regulation**—The ATCase and DHOase activities were not affected by autophosphorylation (data not shown), but there were significant effects on the CPSase activity (Fig. 8, Table II). A steady state kinetic analysis of the CPSase reaction demonstrated that autophosphorylation resulted in a decrease in the $K_m$ for ATP from 3.4 to 2.5 mM and an increase in $V_{max}$ from 3.6 to 5.8 nmol/min. Thus, the apparent second order rate constant, $k_{cat}/K_m$, increases 2.1-fold from 173 to 369 s⁻¹ M⁻¹. ATP does not significantly alter the affinity of the autophosphorylated protein for ATP (Fig. 8B, Table II), but it reduces the $V_{max}$ and increases the cooperativity to a greater extent than that observed for unphosphorylated CAD. When the saturation curve was carried out in the presence of 200 μM PRPP (Fig. 8C, Table II), the $K_m$ for ATP and the $V_{max}$ when expressed as the percentage increase, were about the same for phosphorylated and unphosphorylated CAD.

**Sensitivity to Allosteric Effectors**—The CPSase activity of phosphorylated and unphosphorylated CAD was measured as a function of the concentration of the allosteric ligands (Fig. 9, Table III). Autophosphorylation increased UTP inhibition. There was no significant change in the apparent affinity for UTP judging from the concentration of the nucleotide required to produce half-maximal inhibition. However, the maximum inhibition increased from 49 to 91% when the protein was phosphorylated. Thus, whereas the affinity is about the same, UTP is a much more effective inhibitor of the autophosphoryl-
ated protein. Although PRPP appears to activate the enzyme to a greater extent because of the higher activity of the autophosphorylated protein (Fig. 9B), when expressed on a percentage basis (Table III), PRPP was found to be a less effective activator of phosphorylated CAD. The apparent $K_a$ for PRPP decreased by 40%, indicating a slight increase in apparent affinity for the autophosphorylated protein, but there was a 2-fold reduction in the maximum activation. Thus, PRPP does not activate phosphorylated CAD as efficiently at saturating PRPP concentrations.

**DISCUSSION**

Incubating purified CAD with ATP resulted in its phosphorylation, a phenomenon that was noticed previously by us and others (17) but was never closely examined. For example, Carey et al. (17) suggested that the phosphate incorporation (0.8 mol of P/mol of CAD) that occurred upon incubation of purified CAD with ATP in the absence of PKA could be due to autophosphorylation or to the presence of a contaminating kinase. These authors could distinguish the background phosphate incorporation from PKA-mediated phosphorylation and make appropriate corrections. In this study, we find that phosphorylation occurred reproducibly with the stoichiometric incorporation of 1 mol of phosphate into a specific CAD threonine residue. The reaction has all of the earmarks of a kinase-catalyzed reaction: 1) the $\gamma$-phosphoryl group of ATP was transferred to a threonine residue on the protein; 2) other nucleotide triphosphates did not serve as substrates; 3) ADP appeared to be a competitive inhibitor; 4) the reaction was Mg$^{2+}$-dependent; 5) it was sensitive to the Ser/Thr-specific protein phosphatase I; 6) it was abolished by the ATP analog, FSBA; and 7) it required the native CAD tertiary structure. The observation that phosphorylation occurred when CAD was denatured, purified by SDS-polyacrylamide gel electrophoresis, transferred onto a nitrocellulose membrane, and renatured strongly suggests that...
the reaction resulted from an intrinsic kinase activity of CAD itself and was not the result of contaminants.

Autophosphorylation was initially detected using antibodies directed against Thr(P). These antibodies are quite specific and can, for example, clearly distinguish between MAPK-mediated phosphorylation of Thr456 and the phosphorylation of Ser1406 and Ser1859 by PKA (24). Mass spectrometry analysis of CAD tryptic digests detected only two major phosphopeptides. Phosphopeptide 2 contained Ser1859, the PKA phosphorylation site (site 2) previously identified (17) in the linker connecting the ATC and DHO domains (Fig. 1A). We can rule out phosphopeptide 2 as the target of autophosphorylation based on the observation that phosphorylation of Ser1859 has no effect on CAD function. A mutational analysis (19, 43) involving deletion of PKA site 2 and replacement of site 1 and site 2 serines with alanine residues, individually and together, clearly showed

| Protein | Effectors | $K_{\text{ATP}}$ | $v_{\text{max}}$ | $n$ |
|---------|-----------|-----------------|-----------------|-----|
| CAD     | None      | 3.37 ± 1.17     | 3.59 ± 0.67     | 1.32 ± 0.26 |
| CAD     | 4 mM UTP  | 4.09 ± 0.39     | 1.33 ± 0.10     | 1.85 ± 0.15 |
| CAD     | 200 μM PRPP | 0.46 ± 0.10 | 4.78 ± 0.05 | 1.15 ± 0.04 |
| P-CAD   | None      | 2.53 ± 0.31     | 5.78 ± 0.36     | 1.35 ± 0.12 |
| P-CAD   | 4 mM UTP  | 2.27 ± 0.21     | 1.36 ± 0.09     | 2.54 ± 0.45 |
| P-CAD   | 200 μM PRPP | 0.45 ± 0.04 | 7.82 ± 0.25 | 1.11 ± 0.13 |

* CAD refers to the unphosphorylated protein, and P-CAD represents autophosphorylated CAD.

The $K_i$ and $K_a$ are apparent affinity constants that correspond to the concentration of the allosteric effector that produced the half-maximal effect when assayed with 1.5 mM ATP. These constants, as well as $I_{\text{max}}$ and $A_{\text{max}}$, were obtained by a least squares fit of the data in Fig. 9 to a simple binding isotherm.

This table shows the steady state kinetics of unphosphorylated and autophosphorylated CAD CPSase.

This table shows the allosteric regulation of unphosphorylated and autophosphorylated CAD.

**FIG. 8.** CPSase ATP saturation curves of phosphorylated and unphosphorylated CAD. The ATP saturation curves for the CPSase activity of unphosphorylated (○) and autophosphorylated (●) CAD were measured in the absence of ligands (A) and in the presence of 4 mM UTP (B) and 200 μM PRPP (C). The assays contained 1.5 mM ATP; a 2 mM excess of MgCl₂ over the total concentration of ATP, UTP, and PRPP; and 52 μM CAD.

**FIG. 9.** Sensitivity of phosphorylated and unphosphorylated CAD to allosteric effectors. The CPSase activity of unphosphorylated (○) and phosphorylated (●) CAD was assayed at a concentration of 1.5 mM ATP in the presence of various concentrations of UTP (A) and PRPP (B).

The $K_i$ and $K_a$ are apparent affinity constants that correspond to the concentration of the allosteric effector that produced the half-maximal effect when assayed with 1.5 mM ATP. These constants, as well as $I_{\text{max}}$ and $A_{\text{max}}$, were obtained by a least squares fit of the data in Fig. 9 to a simple binding isotherm.
The active sites on CPS.A and CPS.B that catalyze the ATP-dependent partial reactions involved in carbamoyl phosphate synthesis are also responsible for autophosphorylation. The reaction of CAD with the ATP analog FSBA (42) covalently modifies residues at both active sites and abolishes both carbamoyl phosphate synthesis and, as shown here, autophosphorylation. The ATP saturation curve for the autophosphorylation reaction was hyperbolic and gave a \( K_m \) of 0.33 mM, a value that is low compared with the \( K_m \) for ATP for carbamoyl phosphate synthesis (3.4 mM). However, the separately cloned CAD CPS.A and CPS.B domains (41) exhibited low \( K_m \) values, 0.48 and 0.24 mM, respectively, that may be more representative of the intrinsic ATP affinity of the subdomains. We do not as yet know whether autophosphorylation is catalyzed by CPS.A or CPS.B or both. The phosphorylated residue is appreciably closer to the active site of CPS.B (14 Å between the Thr hydroxyl group and the γ-phosphate of ATP) than to the active site of CPS.A (41 Å). However, an examination of the model structure (Fig. 10) did not show an unobstructed path for phosphoryl transfer between the CPS.B active site and Thr1037 on the same chain, and, as discussed below, an intermolecular phosphoryl transfer can occur.

Autophosphorylation clearly depends on CAD concentration, although the reaction does not conform to second order kinetics. It is likely that autophosphorylation is in part intramolecular (cis) and in part intermolecular (trans). The intermolecular, concentration-dependent component of the reaction, probably involves phosphoryl transfer between CAD molecules. The fundamental oligomeric unit of CAD is a trimer, presumably held together by strong interactions known to exist between ATCase domains. The trimers dimerize to form hexamers (45), the predominate oligomeric form. Thus, intramolecular or cis-phosphorylation could in principle occur within a single polypeptide chain or between adjacent polypeptides within the hexamer. The model structure favors interchain phosphoryl transfer, since phosphoryl transfer to Thr1037 from the catalytic site of either CPS.A or CPS.B would require appreciable changes in the conformation of the protein. Further preliminary evidence for intermolecular phosphoryl transfer was the observation that CAD can phosphorylate bovine serum albumin. If this observation survives scrutiny, CAD could be classified as a protein kinase, although whether there are other more physiologically significant targets remains to be discovered.

Autophosphorylation occurred whether or not the enzyme was catalyzing carbamoyl phosphate synthesis. Although CPSase has a modest turnover number (\( k_{cat} = 0.58 \text{ s}^{-1} \)), the rate of carbamoyl phosphate synthesis is much more rapid than the rate of autophosphorylation. Under typical conditions used in this study, approximately half of the Thr1037 sites were phosphorylated during the course of 1 h. During this same time period, the enzyme would have undergone 2100 cycles of catalysis. Thus, compared with catalysis, autophosphorylation is a relatively rare event. However, once phosphorylated, there were significant changes in CPSase function. The fundamental oligomeric unit of CAD is a trimer, presumably held together by strong interactions known to exist between ATCase domains. The trimers dimerize to form hexamers (45), the predominate oligomeric form. Thus, intramolecular or cis-phosphorylation could in principle occur within a single polypeptide chain or between adjacent polypeptides within the hexamer. The model structure favors interchain phosphoryl transfer, since phosphoryl transfer to Thr1037 from the catalytic site of either CPS.A or CPS.B would require appreciable changes in the conformation of the protein. Further preliminary evidence for intermolecular phosphoryl transfer was the observation that CAD can phosphorylate bovine serum albumin. If this observation survives scrutiny, CAD could be classified as a protein kinase, although whether there are other more physiologically significant targets remains to be discovered.
Thus, the functional consequences of autophosphorylation are clearly distinct from the changes in allosteric regulation produced by these other kinases.

A potential practical consideration is the influence of autophosphorylation on carbamoyl phosphate synthetase assays. The assays are usually carried out for short periods of time (15–30 min) at 37 °C at variable ATP concentrations (0.5–10 mM) and CAD concentration that is generally quite low (typically 0.025 μg/μl). The data reported here (Fig. 6) suggests that during a typical assay period, less than 2% of the CAD would be phosphorylated. This conclusion was supported by assays conducted for variable assay times from 5 min to 2 h at 37 and 30 °C (data not shown). The time course was linear, indicating that the catalytic activity was unaffected by the time of exposure to ATP. Thus, the autophosphorylation reaction would not be expected to significantly perturb the assay results or to have distorted previously published kinetic studies. On the other hand, CAD in cell extracts exhibits a variable degree of sensitivity to allosteric effectors that may be attributed in part to autophosphorylation.

Autophosphorylation of protein kinases is usually believed to have an important regulatory role (26), although there are cases where the physiological significance is not apparent. Autophosphorylation frequently increases the response to allosteric ligands and modulates the interaction of the protein with other protein partners. An intriguing observation that remains to be followed up is that the rate, but not the maximum extent, of autophosphorylation is significantly reduced in the presence of PKA (Fig. 3B). Thus, there may be differences in the effectiveness of phosphorylated and unphosphorylated CAD to serve as a substrate for these other kinases. This effect would be especially interesting, since the functional consequences of autophosphorylation counter those of MAPK and PKA phosphorylation. Studies are planned to determine whether autophosphorylation occurs in vivo and whether MAPK and PKA phosphorylation influences autophosphorylation and vice versa. The CAD complex, which governs the supply of pyrimidine nucleotides, is regulated by an elaborate interplay of allosteric control and phosphorylation mediated by two central signaling cascades. Autophosphorylation introduces another fascinating level of complexity into the CAD regulatory network.

Acknowledgment—We thank Dr. Vasily Studitsky for use of the phosphorimaging system.

REFERENCES
1. Mori, M., Ishida, H., and Tatibana, M. (1975) Biochemistry 14, 2622–2630
2. Shoaf, W. T., and Jones, M. E. (1973) Biochemistry 12, 4039–4051
3. Coleman, P., Suttle, D., and Stark, G. (1977) J. Biol. Chem. 252, 6379–6385
4. Meister, A. (1989) Adv. Enzymol. Relat. Areas Mol. Biol. 62, 315–374
5. Post, L. E., Post, D. J., and Rauschel, F. M. (1990) J. Biol. Chem. 265, 774–7747
6. Nyunoya, H., and Lusty, C. J. (1983) Proc. Natl. Acad. Sci. U. S. A. 80, 4629–4633
7. Chen, J.-J., and Jones, M. E. (1979) J. Biol. Chem. 254, 2697–2704
8. Tatibana, M., and Ito, K. (1969) J. Biol. Chem. 244, 5903–5913
9. Hager, S. E., and Jones, M. E. (1967) J. Biol. Chem. 242, 5667–5673
10. Hager, S. E., and Jones, M. E. (1967) J. Biol. Chem. 242, 5674–5680
11. Levine, R. L., Hoogenraad, N. J., and Kretschmer, N. (1971) Biochemistry 10, 3694–3699
12. Tatibana, M., and Shigesada, K. (1972) Biochem. Biophys. Res. Commun. 46, 491–497
13. Maltby, M. I., Grayson, D. R., and Evans, D. R. (1980) J. Biol. Chem. 255, 11372–11380
14. Shaw, S. M., and Carrey, E. A. (1992) Eur. J. Biochem. 207, 957–963
15. Liu, X., Guy, H. I., and Evans, D. R. (1994) J. Biol. Chem. 269, 27747–27755
16. Sahay, N., Guy, H. I., Xin, L., and Evans, D. R. (1998) J. Biol. Chem. 273, 31195–31202
17. Carrey, E. A., Campbell, D. G., and Hardie, D. G. (1985) EMBO J. 4, 3735–3742
18. Irvine, H. S., Shaw, S. M., Paton, A., and Carrey, E. A. (1997) Eur. J. Biochem. 247, 1063–1073
19. Guy, H. I., and Evans, D. R. (1994) Adv. Exp. Med. Biol. 370, 729–733
20. Banerjee, L. C., and Davidson, J. N. (1997) Somat. Cell. Mol. Genet. 23, 37–49
21. Graves, L. M., Guy, H. I., Kozielowski, P., Huang, M., Lazarowski, E., Pope, R. M., Collins, M. A., Dahlstrand, E. N., Earp, H. S., III, and Evans, D. R. (2000) Nature 403, 328–332
22. Cobb, M. H., and Goldsmith, E. J. (1995) J. Biol. Chem. 270, 14843–14846
23. Lewis, T. S., Shapiro, P. S., and Ahn, N. G. (1998) Adv. Cancer Res. 74, 49–139
24. Sigoillot, F. D., Evans, D. R., and Guy, H. I. (2002) J. Biol. Chem. 277, 15745–15751
25. Hunter, T. (1987) Cell 50, 823–829
26. Smith, J. A., Francis, S. H., and Corbin, J. D. (1993) Mol. Cell. Biochem. 127, 51–70
27. Fernandez, R., Herrero, P., Fernandez, E., Fernandez, T., Lopez-Boado, Y., and Moreno, F. (1998) J. Gen. Microbiol. 134, 2493–2498
28. Heinrich, K., Otto, A., Behlke, J., Rush, J., Wenzel, K., and Kriegel, T. (1997) Biochemistry 36, 1960–1964
29. Hemmer, W., Wurzer-Graves, E., Frank, G., Wallmann, T., and Furter, R. (1995) Biochim. Biophys. Acta 1251, 81–90
30. Guillo, F., Rubino, S. D., Markovitsa, R. S., Kinney, D. M., and Lusty, C. J. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 8304–8308
31. Lowry, O., Rosenbrough, N., Farr, A., and Randall, R. (1951) J. Biol. Chem. 193, 265–275
32. Purcarro, C., Herve, G., Ladjimi, M. M., and Cunin, R. (1997) J. Bacteriol. 179, 4143–4157
33. Guy, H. I., and Evans, D. R. (1994) J. Biol. Chem. 269, 23808–23816
34. Grayson, D. R., Pudgett, R. A., and Stark, G. R. (1979) J. Biol. Chem. 254, 8679–8689
35. Purcarro, C., Herve, G., Ladjimi, M. M., and Cunin, R. (1997) J. Bacteriol. 179, 4143–4157
36. Guy, H. I., and Evans, D. R. (1994) J. Biol. Chem. 269, 23808–23816
37. Guillou, F., Rubino, S. D., Markovitsa, R. S., Kinney, D. M., and Lusty, C. J. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 8304–8308
38. Lowry, O., Rosenbrough, N., Farr, A., and Randall, R. (1951) J. Biol. Chem. 193, 265–275
39. Guy, H. I., and Evans, D. R. (1995) J. Biol. Chem. 269, 15840–15849
40. Laemmli, U. (1970) Nature 227, 680–685
41. Prescott, L. M., and Jones, M. E. (1969) Anal. Biochem. 32, 408–419
42. Pastra-Landis, S. C., Foote, J., and Kantrowitz, E. R. (1981) Anal. Biochem. 118, 358–363
43. Ferrell, J. E., Jr., and Martin, G. S. (1989) J. Biol. Chem. 264, 20723–20729
44. Guy, H. I., and Evans, D. R. (1996) J. Biol. Chem. 272, 13762–13769
45. Kim, H. S., Lee, L., and Evans, D. R. (1991) Biochemistry 30, 10322–10329
46. Kemling, N. (1994) Regulation of the multifunctional protein, CAD. Ph.D. dissertation, Wayne State University
47. Bradzok, B. L., Mullins, L. S., Rauschel, F. M., and Righthart, G. D. (1992) Biochemistry 31, 2309–2316
48. Lee, L., Kelly, R. E., Pastra-Landis, S. C., Evans, D. R. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 6802–6806
Autophosphorylation of the Mammalian Multifunctional Protein That Initiates de Novo Pyrimidine Biosynthesis
Frederic D. Sigoillot, David R. Evans and Hedeel I. Guy

J. Biol. Chem. 2002, 277:24809-24817. doi: 10.1074/jbc.M203512200 originally published online May 1, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M203512200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 44 references, 19 of which can be accessed free at http://www.jbc.org/content/277/27/24809.full.html#ref-list-1