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Identification of the ATP Binding Sites of the Carbamyl Phosphate Synthetase Domain of the Syrian Hamster Multifunctional Protein CAD by Affinity Labeling with 5′-[*p*-(Fluorosulfonyl)benzoyl]adenosine†

Hyesook Kim,‡,§ Lana Lee,‖ and David R. Evans*,‡
Department of Biochemistry, Wayne State University School of Medicine, 540 East Canfield Street, Detroit, Michigan 48201, and Department of Chemistry and Biochemistry, University of Windsor, Windsor, Ontario, Canada N9B 3P4
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ABSTRACT: The ATP analogue 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine (FSBA) was used to chemically modify the ATP binding sites of the carbamyl phosphate synthetase domain of CAD, the multifunctional protein that catalyzes the first steps in mammalian pyrimidine biosynthesis. Reaction of CAD with FSBA resulted in the inactivation of the ammonia- and glutamine-dependent CPSase activities but had no effect on its glutaminase, aspartate transcarbamylase, or dihydroorotase activities. ATP protected CAD against inactivation by FSBA whereas the presence of the allosteric effectors UTP and PRPP afforded little protection, which suggests that the ATP binding sites were specifically labeled. The inactivation exhibited saturation behavior with respect to FSBA with a K_I of 0.93 mM. Of the two ATP-dependent partial activities of carbamyl phosphate synthetase, bicarbonate-dependent ATPase was inactivated more rapidly than the carbamyl phosphate dependent ATP synthetase, which indicates that these partial reactions occur at distinct ATP binding sites. The stoichiometry of [^{14}C]FSBA labeling showed that only 0.4–0.5 mol of FSBA/mol of protein was required for complete inactivation. Incorporation of radiolabeled FSBA into CAD and subsequent proteolysis, gel electrophoresis, and fluorography demonstrated that only the carbamyl phosphate synthetase domain of CAD is labeled. Amino acid sequencing of the principal peaks resulting from tryptic digests of FSBA-modified CAD located the sites of FSBA modification in regions that exhibit high homology to ATP binding sites of other known proteins. Thus CAD has two ATP binding sites, one in each of the two highly homologous halves of the carbamyl phosphate domain which catalyze distinct ATP-dependent partial reactions in carbamyl phosphate synthesis.

In mammalian cells, glutamine-dependent carbamyl phosphate synthetase (CPSase;[1] EC 6.3.5.5), the first and rate-limiting step in de novo pyrimidine biosynthesis, is associated with the multifunctional enzyme CAD (Jones, 1980); each 240-kDa subunit of this oligomeric protein (Shoaf & Jones, 1973; Mori et al., 1975; Coleman et al., 1977; Lee et al., 1985) also catalyzes the second and third reactions in pyrimidine biosynthesis, aspartate transcarbamylase (ATCase; EC 2.1.3.2) and dihydroorotase (DHOase; EC 3.5.2.3). Controlled proteolysis studies indicate that CAD consists of several domains, each with a distinct function (Mally et al., 1981; Davidson et al., 1981; Grayson et al., 1985); both the ATCase and DHOase domains of CAD have been isolated and sequenced (Grayson & Evans, 1983; Kelly et al., 1986; Shigesada et al., 1985; Major et al., 1989; Simmer et al., 1989, 1990a). The primary sequence and structural organization of the CAD CPSase domain are very similar to those of other monofunctional CPSases (Simmer et al., 1990b). The 120-kDa synthetase domain is comprised of two highly homologous subdomains which we have designated CPS.A and CPS.B.

Carbamyl phosphate synthetase catalyzes the ATP-dependent formation of carbamyl phosphate, a key intermediate in pyrimidine and arginine biosynthesis (Jones, 1980). The observation that *Escherichia coli* CPSase catalyzes two partial reactions, a bicarbonate-dependent ATPase and carbamyl phosphate dependent ATP synthesis, in conjunction with other kinetic and mechanistic studies (Anderson & Meister, 1965, 1966; Powers & Meister, 1976, 1978a,b) showed that carbamyl phosphate synthesis occurs in four steps with the formation of two intermediates, carboxyl phosphate and carbamate. The physiological substrate is glutamine, although ammonia can also directly serve as a nitrogen donor:

$$\text{glutamine} \rightarrow \text{glutamate} + NH_3$$
$$ATP + HCO_3^- \rightarrow ADP + \text{carboxy phosphate}$$
$$\text{carboxy phosphate} + NH_3 \rightarrow \text{carbamate}$$
$$\text{carbamate} + ATP \rightarrow \text{carbamyl phosphate}$$

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* To whom correspondence should be addressed.
‡ Wayne State University School of Medicine.
§ Current address: Institute of Chemical Toxicology, Wayne State University, Detroit, MI 48201.
‖ University of Windsor.

[1] Abbreviations: ATCase, aspartate transcarbamylase; CAD, the protein having glutamine-dependent carbamyl phosphate synthetase, aspartate transcarbamylase, and dihydroorotase activities; CPSase, carbamyl phosphate synthetase; DHOase, dihydroorotase; EDTA, ethylenediaminetetraacetic acid; FSBA, 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine; GLN, glutamine; Me_2SO, dimethyl sulfoxide; TFA, trifluoroacetic acid; TLC, thin-layer chromatography; TPCK, L-1-(tosylamino)-2-phenylethyl chloromethyl ketone.

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Thus the overall reaction requires 2 molecules of ATP. One ATP is required for the formation of carboxy phosphate; the other is required for the phosphorylation of carbamate to form carbamyl phosphate.

Sequencing of *E. coli*, yeast, and rat mitochondrial CPSase (Nyunoya & Lusty, 1983; Nyunoya et al., 1985; Lusty et al., 1983) showed that the amino and carboxyl halves of the 120-kDa synthetase domain are highly homologous, which may have occurred by an ancestral gene duplication and fusion. The CAD CPS domain has a similar structural organization (Simmer et al., 1990b). Two distinct ATP binding sites have been demonstrated in *E. coli* CPSase (Powers et al., 1977; Powers & Meister, 1978a) and CPSase I (Rubio et al., 1979; Powers-Lee & Corina, 1987). The location of the ATP binding sites has been proposed on the basis of the presence of short sequences homologous to those found in the ATP binding sites of other proteins (Lusty et al., 1983; Nyunoya & Lusty, 1983; Nyunoya et al., 1985; Powers-Lee & Corina, 1987; Souciet et al., 1989). Larger regions postulated to correspond to ATP binding subdomains have been assigned in *E. coli* CPSase (Takai et al., 1988) and CAD (Simmer et al., 1990b) on the basis of strong homology to yeast pyruvate carboxylase (Lim et al., 1988) and chicken acetyl-CoA carboxylase (Takai et al., 1988).

The affinity label 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine (FSBA) was used to probe the ATP binding sites of the carbamyl phosphate synthetase domain of CAD. A recent review documents the extensive applications of FSBA to the study of nucleotide binding sites of a number of enzymes (Colman, 1989). A difference in the rate of inactivation of the partial reactions involved in carbamyl phosphate synthesis could in principle be used to distinguish between the two ATP binding sites of the enzyme, but the ATP-dependent reactions catalyzed by *E. coli* CPSase are simultaneously inactivated by FSBA (Boettcher & Meister, 1980). However, in this study, we find that FSBA differentially affects the bicarbonate-dependent ATPase and carbamyl phosphate ATP synthetase activities. The sites of FSBA modification sites were found to be located within the putative ATP binding subdomains of CAD.

Experimental Procedures

CAD Purification. CAD was purified from an overproducing, SV-40 transformed Syrian hamster cell line (165-23) generously provided by Dr. George R. Stark (Imperial Cancer Research Fund, London, England). The cells were grown by the method of Kempe et al. (1976) at the Massachusetts Institute of Technology Cell Culture Center, Cambridge, MA, and removed from the surface incubation in 0.5 mM EDTA in phosphate-buffered saline at 37 °C for 5 min. CAD was isolated by the method of Coleman et al. (1977) and stored at −70 °C at a concentration of 3 mg/mL in 20 mM Tris, 50 mM KCl, 4 mM glutamine, 4 mM aspartate, 0.1 mM EDTA, 1 mM dithiothreitol, 5% glycerol, and 30% dimethyl sulfoxide, pH 7.4. Concentrations of CAD solutions were determined by the method of Bradford (1976) using the Bio-Rad protein assay (Bio-Rad).

Ammonia- and Glutamine-Dependent CPSase. The ammonia- and glutamine-dependent CPSase activities were measured by previously published methods (Mally et al., 1980; Coleman et al., 1977) which quantitate the incorporation of radioactively labeled [^{14}C]sodium bicarbonate into carbamyl phosphate. The concentration of the nitrogen donor was either 100 mM NH_4Cl or 5 mM glutamine. Alternatively, carbamyl aspartate, derived from carbamyl phosphate by the endogenous ATCase activity of CAD, was measured colorimetrically (Prescott & Jones, 1969) whenever the enzyme was chemically modified by a potentially interfering radiolabel.

Bicarbonate-Dependent ATPase Assay. The partial reaction of bicarbonate-dependent ATPase was monitored by TLC methods in which the conversion of [^{14}C]ATP into [^{14}C]ADP could be distinguished and quantitated. The assay mixture consisted of 50 mM Tris-HCl, 2 mM $MgCl_2$, 100 mM KCl, 25 mM sodium bicarbonate, 1 mM ATP, and 5–25 μg of CAD, pH 7.8. The reaction at 37 °C was initiated by the addition of 10 μL of 5 mM, 2.0 mCi/mmol [^{14}C]ATP (New England Nuclear) to 40 μL of the reaction mixture. Poly(ethyleneimine)–cellulose plates MN300, 0.1 mm (Brinkmann Instruments), were washed with water, air-dried, and stored at 4 °C. The TLC plate was spotted with 5 μL of 5 mM ADP and 5 μL of 5 mM ATP. After 10-min reaction time, the reaction was quenched by the application of 10 μL of the reaction mixture onto the TLC plate. The plate was air-dried for 20 min and then developed in 1 M LiCl for 90 min as previously described (Mace & Alberts, 1984). The dried TLC plate was autoradiographed with Kodak X-OMAT AR X-ray film overnight. The ATP and ADP spots were visualized under UV light, cut out, and placed into scintillation vials. After 1 mL of water was added to each vial, they were shaken overnight. Ten milliliters of ACS scintillation fluid (Packard Corp.) was then added to each vial, and the samples were counted in a Packard Tri-Carb scintillation counter. The bicarbonate-dependent ATPase activity was calculated by subtracting the ATPase activity of controls which lacked bicarbonate but were otherwise identical.

Carbamyl Phosphate Dependent ATP Synthetase Assay. This assay monitors the conversion of [^{14}C]ADP into [^{14}C]-ATP by TLC methods. The assay mixture consisted of 50 mM Tris-HCl, 2 mM $MgCl_2$, 100 mM KCl, 10 mM carbamyl phosphate, 1 mM ADP, and 5–25 μg of CAD. The reaction was initiated by the addition of 10 μL of 5 mM [^{14}C]ADP (2.0 mCi/mmol) to 40 μL of reaction mixture. After 10 min, the reaction was terminated and the assay processed as described above. Control experiments showed that there was no significant formation of ATP in the absence of carbamyl phosphate.

Modification of CAD with FSBA. CAD was modified with 0.3–4.0 mM FSBA at 37 °C. The reaction mixture consisted of 1.25 mg/mL CAD, 56 mM Tris-HCl, 110 mM KCl, 0.8 mM aspartate, 0.02 mM EDTA, 26% (v/v) Me_2SO, 1% (v/v) glycerol, and 2 mM $MgCl_2$, pH 8.2. At selected time intervals, aliquots of the reaction mixture were removed and assayed for enzymatic activity (see above). In selected experiments, CAD or CAD digests were reacted with radiolabeled FSBA. Solutions of [^{14}C]FSBA (43.2 and 53.6 mCi/mmol) in 95% ethanol and 5% water (New England Nuclear) were lyophilized and redissolved to the appropriate concentration in Me_2SO. [^{14}C]FSBA was radiolabeled on carbon 8. The reaction was carried out as described above. To terminate the reaction, unreacted [^{14}C]FSBA was removed using the buffer exchange method (Penefsky, 1977). Biphasic progress curves were resolved into two-exponential curves by graphical approximation.

Controlled Proteolysis. CAD was digested with trypsin using a ratio of protein to protease of 100 to 1 as described previously (Mally et al., 1981).

Peptide Mapping. TPCK-treated trypsin (Worthington) was dissolved in 50 mM NH_4HCO_3 at a concentration of 1 mg/mL. The concentration was determined by measuring the

absorbance at 280 nm using an extinction coefficient of 1.43 $(mg/mL)^{-1}$ cm^{-1}. CAD was modified with 0.4–2 mM FSBA for 30 min as described above. The FSBA-modified CAD was reduced, alkylated (Konigsberg, 1972), and then dialyzed against phosphate buffer and 10 mM EDTA, pH 7.5. FSBA-modified CAD was digested with trypsin at a ratio of protease to protein of 1 to 100 (w/w) for 24 h at 37 °C as previously described (Kelly et al., 1986). A second aliquot of trypsin, at the same trypsin to protein ratio, was introduced for the next 24-h incubation period. The digest was lyophilized, and the peptides were dissolved in 3 M guanidine hydrochloride in 5% acetonitrile and 0.1% trifluoroacetic acid, applied to a C-18 μBondapak (Waters) reverse-phase column, and eluted with a linear gradient of 5–35% or 5–65% acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 1 mL/min for 4 h at room temperature. The column effluents of digests of both labeled and unlabeled CAD were monitored at 214 and 254 nm with a Waters 490 UV detector. For isolation of [^{14}C]FSBA-labeled peptides, the modified protein (2.5 nmol) was denatured with 0.32% TFA (v/v), precipitated with 10 volumes of cold acetone, washed three times at room temperature with acetone, digested with trypsin [protein/protease = 125 (w/w)] for 16 h, and then fractionated by HPLC as described above. This procedure for digesting the protein gave comparable results to the more exhaustive conditions described above.

Analysis of Isolated Peptides. Protein sequence determination was performed by the Macromolecular Core Facility at the School of Medicine, Wayne State University. Amino acid microsequence analysis was obtained by automated Edman chemistry on an Applied Biosystems gas-phase sequenator (Model 470) with on-line HPLC (Model 120) and a Nelson analytical chromatography data system. The modified residue could not be determined directly because of the lability of the adduct. The entire peptide was not always sequenced, but enough cycles were carried out to unambiguously identify it.

SDS Gel Electrophoresis and Fluorography. Sodium dodecyl sulfate gel electrophoresis was carried out on 1.5-mm gradient slabs which varied from 7.5% to 15% (w/v) acrylamide using the Laemmli buffer system (Laemmli, 1970). Half of the gel was used for protein determination and the other half for fluorography. An identical series of samples were applied to each half of the gel. For protein determination, the gel was stained with Coomassie Brilliant Blue R, destained, and scanned with a Zeineh soft laser scanner (Biomed Instruments, Inc.). The intensity of each band was taken as the area measured by cutting and weighing the densitometer trace. The amount of protein was then calculated using calibration curves obtained by scanning various known amounts of standard proteins run on the same gel. For fluorography, the gel was soaked with gentle rocking for 30 min in Enhance (New England Nuclear) and then for an additional 30 min in water. The gel was dried onto filter paper with a Hoefer gel dryer and then autoradiographed with Kodak X-OMAT AR X-ray film for 3 days. Radioactivity was quantitated by scanning the autoradiograph. Alternatively, the bound FSBA was determined by excising the fragment from the Coomassie Blue stained, dried gel and soaking in 0.25 mL of water. The gel slice was dissolved by adding 1 mL of Protosol and heating at 50 °C for 24 h prior to counting in 10 mL of ACS scintillation fluid (Packard).

Results

Inactivation of CAD with FSBA. FSBA rapidly inactivates both the glutamine- (data not shown) and the ammonia-dependent CPSase activity of CAD. The rate of inactivation

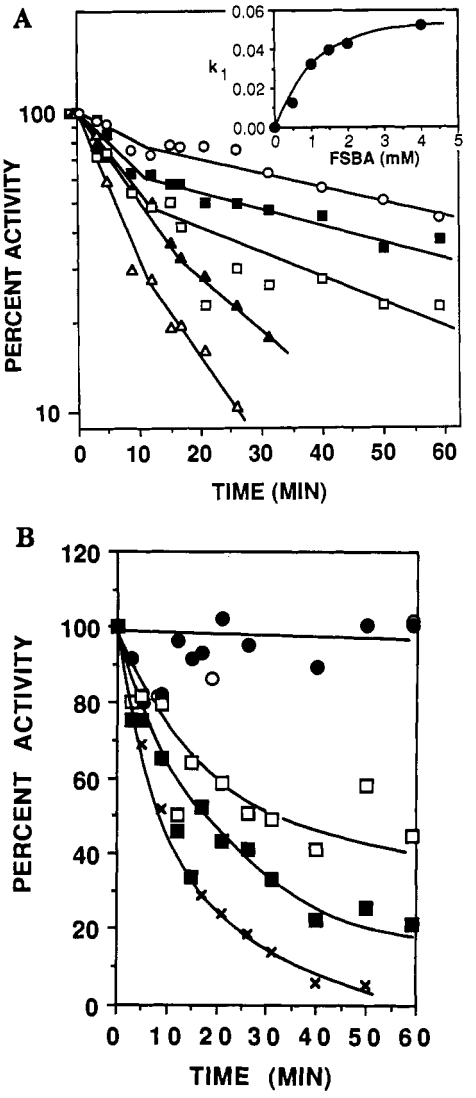

FIGURE 1: Kinetics of the inactivation of the ammonia-dependent CPSase activity of CAD by 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine. (A) Ammonia-dependent carbamyl phosphate synthetase activity of CAD (1 mg/mL) as a function of incubation time of CAD with the following concentrations of FSBA: (○) 0.5 mM; (■) 1 mM; (□) 1.5 mM; (▲) 2 mM; (△) 4 mM. These experiments were carried out at 37 °C in 56 mM Tris-HCl, 110 mM KCl, 0.8 mM aspartate, 0.02 mM EDTA, 26% (v/v) Me_2SO, 1% (w/v) glycerol, and 2 mM $MgCl_2$, pH 8.2. The ammonia-dependent CPSase activity of FSBA-modified CAD was immediately determined after each time point and is expressed as the percent activity of unmodified CAD. The rate constant k_1 for the rapid phase as a function of FSBA concentration is shown in the insert. (B) The effect of Mg-ATP, Mg-UTP, and PRPP on the FSBA inactivation of the ammonia-dependent CPSase activity of CAD was also determined. The reaction was carried out as described above in the presence of the following: (×) no additional ligands, 2 mM FSBA; (○) 10 mM ATP, 0 mM FSBA; (●) 10 mM ATP, 2 mM FSBA; (□) 10 mM UTP, 2 mM FSBA; (■) 1 mM PRPP, 2 mM FSBA.

of the ammonia-dependent CPSase increases with increasing FSBA concentration (Figure 1A), but the progress curves are biphasic, indicating that this is not a simple pseudo-first-order reaction. The curves could be resolved into rapid (k_1) and slow (k_2) exponential phases. Replots of k_1 (Figure 1A, insert), the rate of inactivation for the rapid phase, against the FSBA concentration showed that the inactivation exhibits saturation phenomenon typical of affinity reagents. The K_I was found to be 0.93 mM.

ATP Protection against FSBA Inactivation. The ammonia-dependent carbamyl phosphate synthesis was virtually abolished by reaction with 2 mM FSBA in the absence of

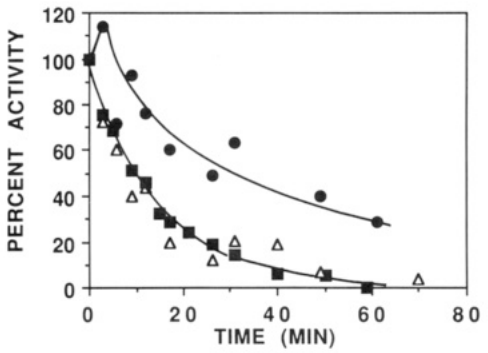

FIGURE 2: Kinetics of inactivation of the overall and partial reactions of the carbamyl phosphate synthetase activity of CAD by reaction with 2 mM FSBA. These experiments were carried out as described under Experimental Procedures. The symbols represent the following: (●) carbamyl phosphate dependent ATP synthetase; (△) bicarbonate-dependent ATPase; (■) ammonia-dependent carbamyl phosphate synthetase.

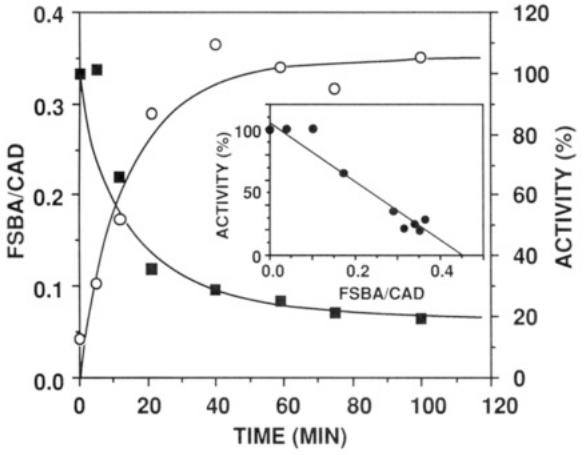

FIGURE 3: Stoichiometry of the reaction of ^{14}C-labeled FSBA and CAD. Inactivation of ammonia-dependent CPSase activity of CAD by the reaction of CAD with 0.4 mM [^{14}C]FSBA was carried out as described under Experimental Procedures: (○) mol of incorporated FSBA/mol of CAD; (■) % residual ammonia-dependent CPSase activity. (Insert) % residual ammonia-dependent CPSase activity of CAD as a function of mol of FSBA incorporated/mol of CAD.

ligands for 1 h (Figure 1B). In contrast, the progress curve for the reaction carried out in the presence of 10 mM ATP was indistinguishable from controls in which CAD was incubated in the absence of FSBA. The presence of either the allosteric inhibitor UTP or the activator PRPP afforded little protection against FSBA inactivation. These results suggested that the FSBA reacted specifically with the ATP binding sites of the CPSase domain of CAD, although the possibility remains that an ATP-induced conformational change may alter the accessibility of a distant reactive residue.

Effects of FSBA on the Partial Reactions of CPSase. The effect of FSBA on the two partial reactions of the CPSase activity of CAD, carbamyl phosphate dependent ATP synthetase and bicarbonate dependent ATPase, was determined as a function of reaction time to establish which sites were modified (Figure 2). The inactivation of the bicarbonate-dependent ATPase coincided with the loss of ammonia-dependent CPSase activity while carbamyl phosphate dependent ATP synthesis was also inactivated but at an appreciably slower rate. The rate of inactivation of each of the partial reactions was also measured as a function of FSBA concentration (data not shown). Both curves exhibited saturation, but the extent of inactivation of the bicarbonate-dependent ATPase at limiting FSBA was about 3-fold greater than that of carbamyl phosphate dependent ATP synthesis. The observed differential effect of FSBA on the two partial reactions of the CPSase activity suggests that FSBA reacts with two separate ATP binding sites at different rates.

Stoichiometry of the Modification of CAD by [^{14}C]FSBA. A time course for the inactivation of the CAD CPSase and the extent of incorporation of the reagent is shown in Figure 3. As the time of reaction increases, there was a corresponding decrease in the activity of the overall ammonia-dependent carbamyl phosphate synthetase reaction. A linear extrapolation of the replot of this data (Figure 3, insert) indicated that the ammonia-dependent CPSase activity was completely abolished when 0.45 mol of FSBA had been incorporated per mole of CAD subunit.

Incorporation of FSBA into Proteolytic Fragments of CAD. Controlled proteolysis of CAD by trypsin cleaves the molecule into a 36-kDa ATCase domain, a 46-kDa DHOase domain, and a 154-kDa fragment which contains the GLN and CPS domains (Figure 4). The 154-kDa species subsequently undergoes a series of cleavages (Figure 4 and the left panel of Figure 5).

When a tryptic CAD digest was reacted with [^{14}C]FSBA, only those fragments containing the CPS domains (200, 154, 140, 80, and 60 kDa) could be detected by fluorography of SDS–polyacrylamide gels (right panel of Figure 5). FSBA was not incorporated into the 36-kDa ATCase domain, 46-kDa DHOase domain, or a 29-kDa fragment which contains 75% of the GLN domain. Densitometric scans of the stained gel and fluorographs (Table I) showed that the extent of modi-

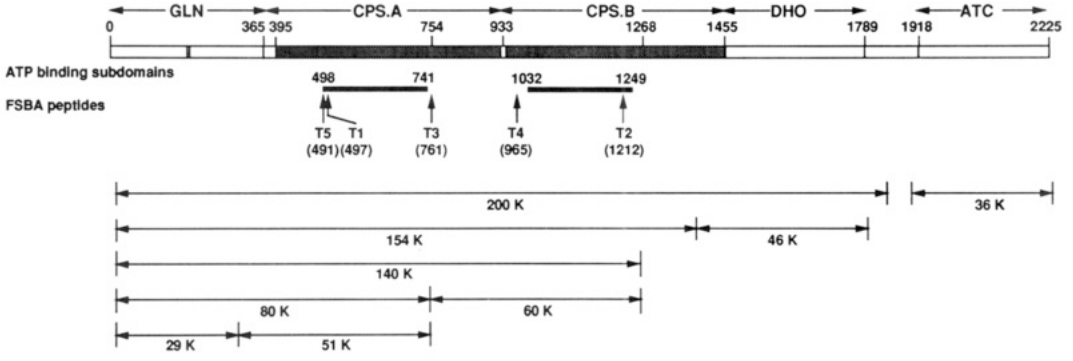

FIGURE 4: Diagram illustrating the relative locations of the CPS.A and CPS.B subdomains of CAD (shaded bar) and the fragments (horizontal arrows beneath the schematic) produced by controlled trypsin digestion. Also indicated are the ATP binding subdomains (solid bars) which have been proposed by an analysis of sequence homologies (Simmer et al., 1990b) and the observed positions of the FSBA-containing tryptic peptide (vertical arrows) of FSBA-modified CAD. The numbering corresponds to the amino acid number of the CAD (Bein et al., 1991). These numbers are higher by 155 than those previously published (Simmer et al., 1990b) to account for the segment at the amino end of CAD which had not been sequenced at the time.

Table I: Incorporation of FSBA into CAD Proteolytic Fragments

ligand	FSBA (pmol)/CAD fragment (pmol)[a]						
	29 kDa	39 kDa	46 kDa	51 kDa	60 kDa	80 kDa	140 kDa
none	0	0.02	0.02	0.11	0.31	0.08	0.65
ATP	0	0.02	0.02	0.10	0.14	0.06	0.16
UTP	0	0.01	0	0.07	0.24	0.07	0.40

[a] The amount of each proteolytic fragment was determined by scanning the Coomassie Blue stained calibrated gel (Figure 5). Radioactivity was quantitated either by excising the band and counting and by scanning the autoradiograph (Figure 5). The values are the average obtained by these two methods.

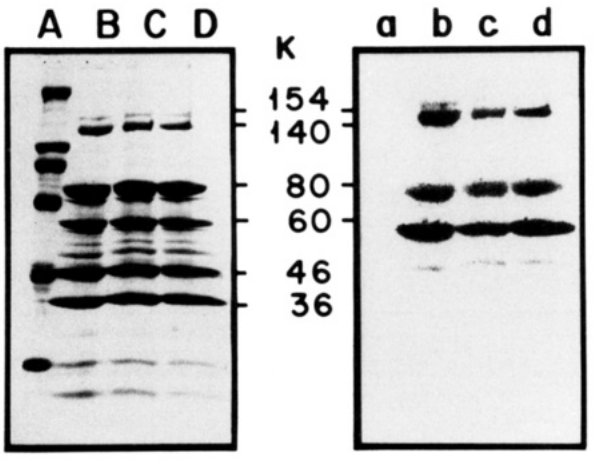

FIGURE 5: Reaction of FSBA with CAD proteolytic fragments. CAD was digested for 35 min at a protein to protease ratio of 100 to 1 (w/w). The digest was then reacted with 0.4 mM [^{14}C]FSBA for 45 min in the presence and absence of nucleotides and then fractionated by electrophoresis in 7.5–15% gradient polyacrylamide gels. The gels were stained with Coomassie Blue (left panel) and fluorographed (right panel). Molecular weight markers (lanes A and a) were myosin (205K), *E. coli* β-galactosidase (116K), rabbit phosphorylase B (97K), bovine serum albumin (66K), ovalbumin (45K), and bovine carbonic anhydrase B (29K); CAD digests reacted with FSBA in the absence of nucleotides (lanes B and b), in the presence of 3.3 mM ATP (lanes C and c), and in the presence of 3.3 mM UTP (lanes D and d).

fication of the 60-kDa fragment was about 4-fold higher than that of the 80-kDa species. The labeling of the 80- and 51-kDa fragments was approximately the same (0.08 and 0.11 pmol of FSBA/pmol of CAD polypeptide, respectively). This result is consistent with the observation that the 29-kDa fragment is unlabeled. When the reaction was carried out in the presence of ATP, FSBA incorporation in the 60-kDa fragment was reduced by about 50%, whereas UTP had a much smaller effect. The major effect of ATP is to reduce the incorporation into the 60-kDa fragment. Since this concentration of ATP should have reduced the rate of FSBA modification 90–95%, the partial protection afforded by the nucleotide in these experiments is probably due to some nonspecific incorporation. Nevertheless, only one radiolabeled peptide was found in each of the 60- and 80-kDa fragments (see below and Table II). Identical results were obtained when CAD was first reacted with FSBA and then cleaved with trypsin; however, the modified 60-kDa fragment is much less resistant to proteolysis and does not accumulate to very high levels.

A kinetic study of FSBA incorporation into the CAD proteolytic fragments (Figure 6A–C) showed that the 60-kDa species initially reacted more rapidly than the 80-kDa species. The time course for the modification of the 140- and 154-kDa fragments was approximately equal to the sum of the incorporation of FSBA into the 80- and 60-kDa fragments at each time point. This result indicated that the rate of FSBA incorporation into CPS.A and CPS.B was not significantly altered by cleavage of the polypeptide chain between the two ATP binding subdomains. As observed in reactions carried out at 2 mM FSBA (Figure 2), 0.3 mM FSBA inactivated

Table II: Sequence of FSBA-Labeled Tryptic Peptides

peptide	sequence[a]	location in CAD sequence: residues	subdomain
	Experiment 1: 2 mM FSBA		
T1[b]	YGVR AFAAR	497–500 517–521	amino end of CPS.A ATP
T2	VIECNV-RX	1212–1218	carboxyl end of CPS.B ATP
T3	SVGEVX-GIG	761–769	near carboxyl end of CPS.A ATP
	Experiment 2: 0.4 mM FSBA[c]		
T4	MGYK	965–968	near amino end of CPS.B ATP
T5	AGVLAR	491–496	amino end of CPS.A ATP

[a] Possible FSBA-labeled residues indicated by boldface type. X refers to an ambiguous residue. [b] T1 contained a mixture of two peptides which mapped close to each other in the CAD sequence. [c] Only two radioactive peaks were detected: T4 had 8852 cpm and T5 had 4316 cpm. The remainder of the counts (9032 cpm) eluted as a low-level background throughout the column profile and may be due to minor nonspecific labeling at many sites.

the bicarbonate-dependent ATPase (Figure 6D), but the lower concentration of the reagent had no measurable effect on the ATP synthetase activity. These results agreed with the kinetics of inactivation of the overall reaction (Figure 1A), which showed that when CAD was reacted with 0.5 mM FSBA for 45 min, 55% of the ammonia-dependent CPSase activity was abolished.

Isolation and Characterization of the Tryptic Peptides of FSBA-Labeled CAD. After reacting CAD for 30 min with 2 mM FSBA, which resulted in a 82% loss of the CPSase activity, the modified protein was denatured and exhaustively digested with trypsin. The resulting tryptic peptides were separated by reverse-phase high-performance liquid chromatography. The elution profile (Figure 7) measured at 214 nm (upper panel) and at 254 nm (lower panel), where the FSBA-labeled peptide absorbs strongly, showed that there were three major modified peaks (T1, T2, and T3). The sequences of the peptides determined by Edman degradation are shown in Table II. In most instances the fractions contained more than one peptide; however, the labeled peptide could always be assigned because the others were found to have originated from regions of the polypeptide that did not react with FSBA. T1 and T3 are located near the amino and carboxyl ends, respectively, of the ATP binding domain of CPS.A, while T2 is located near the carboxyl and of the subdomain of CPS.B. Digests of CAD modified in the presence of 2 mM ATP were also analyzed (data not shown). ATP reduced the labeling of T1 to the greatest extent (33%)[2] but also lowered the incorporation of FSBA into T2 and T3 (15% and 13%, re-

[2] This level of protection is probably reasonable given the relative affinity of ATP and FSBA for the high-affinity ATP binding site. The effect of the substrate on the rate of reaction of the affinity reagent can be estimated using approach devised by Meloche (1967); 2 mM ATP (K_m = 0.5 mM) would reduce the rate of reaction of 2 mM FSBA (K_I = 0.93 mM) by only 56%. In contrast, 10 mM ATP (see Figure 1B) would reduce the rate of the reaction by 87%.

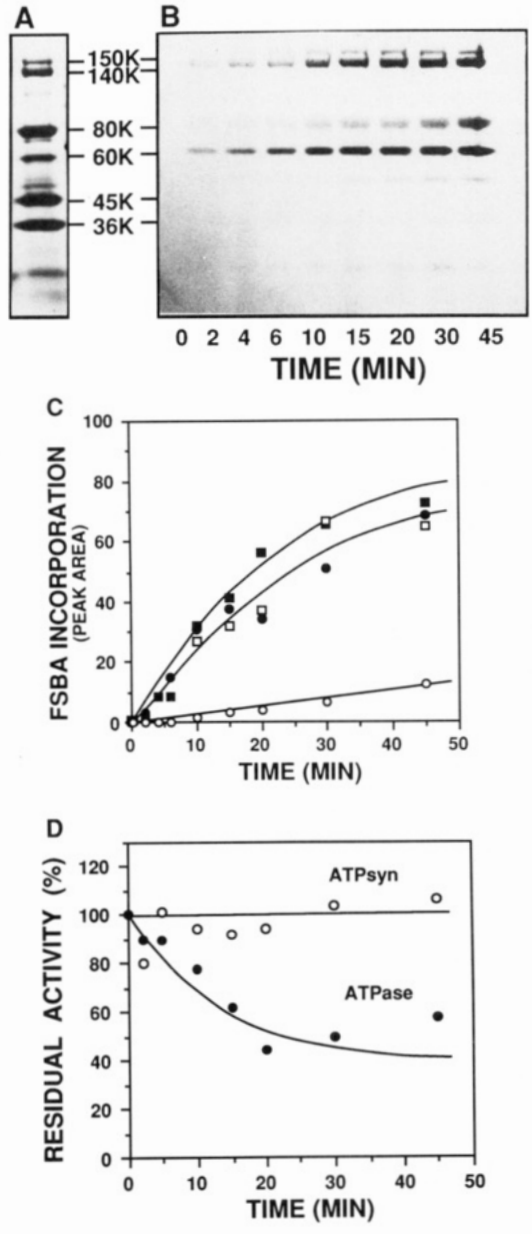

FIGURE 6: Kinetics of FSBA incorporation in CAD proteolytic fragments. A tryptic CAD digest was prepared by digesting 640 μg of CAD with trypsin as described under Experimental Procedures. The digest (0.82 mg/mL) was reacted with 0.3 mM FSBA. At the times indicated 0.020-mL aliquots of the reaction mixture were fractionated by SDS gel electrophoresis. The gel was stained with Coomassie Blue (panel A) and then dried and autoradiographed (panel B). The FSBA incorporation (panel C) into the 60-kDa (●), 80-kDa (○), 140-kDa (■), and 154-kDa (□) fragments was determined by scanning the autoradiograph. The peak areas were corrected for differences in molecular weight and the amount of each species. The bicarbonate-dependent ATPase (●) and carbamyl phosphate dependent ATP synthesis (○) activities were also assayed (panel D) during the course of the reaction.

spectively). When the experiment was repeated using a 5-fold lower concentration of FSBA (0.4 mM), this time using the ^{14}C-labeled reagent and radioactive detection, two labeled peptides were recovered, T4 and T5. The relative amounts of radioactivity incorporated into T4 and T5 are given in Table II. T5 and T1 are contiguous peptides, so that the same region of CPS.A is labeled at both high and low FSBA concentrations. In CPS.B peptides T2 and T3 were not modified; instead only T4, not detected at high FSBA concentrations, was recovered. Edman degradation showed that T4 was located near the amino end of the ATP binding subdomain of CPS.B.

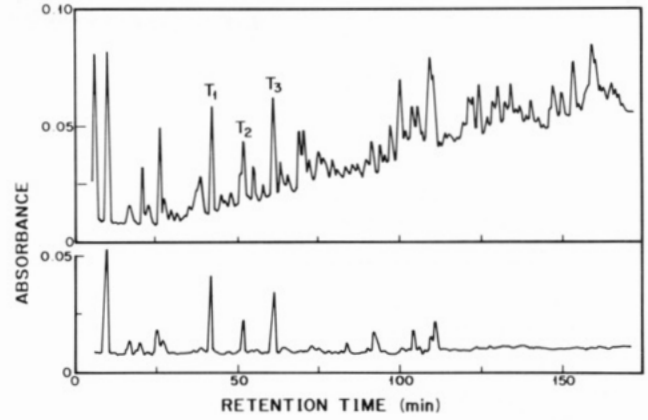

FIGURE 7: Peptide map of FSBA-modified CAD. CAD was reacted with 2 mM FSBA at 37 °C for 30 min; FSBA-modified CAD was then subjected to reductive alkylation and digested with trypsin. Digestion was initiated at 37 °C by the addition of trypsin at a protein to protease ratio of 100 to 1 (w/w). After 24 h, a second equivalent aliquot of trypsin was introduced and the reaction continued for an additional 24 h. The peptides were then lyophilized and fractionated on a C-18 μBondapak column (Waters) with a gradient of 5–35% acetonitrile for 240 min in 0.1% trifluoroacetic acid. The column effluents were read at 214 nm to monitor all eluted peptides (top panel) and at 254 nm to monitor all FSBA-modified peptides (bottom panel). The major peak in the 254-nm map at 10 min as well as the minor unidentified peaks were present in control digests (not shown) of unmodified CAD.

Discussion

The reaction of CAD with the adenosine analogue FSBA appears to be specific, since (1) the reaction exhibits saturation kinetics, (2) CPSase activity is completely abolished by the incorporation of less than 1 mol of the reagent per polypeptide chain, (3) only the CPS.A and CPS.B subdomains of CAD react with the reagent, and (4) ATP completely blocks FSBA inactivation.

The inactivation kinetics are biphasic, and the measurements of the stoichiometry of the reaction indicated that the incorporation of 0.5 mol of FSBA is sufficient to completely inactivate the enzyme. We do not understand the underlying mechanistic reasons for this unusual behavior, but similar observations were made when *E. coli* CPSase was reacted with FSBA (Boettcher & Meister, 1980). These effects may be an indication of interactions between ATP binding sites on different subunits within the oligomer. CAD is a hexamer (Lee et al., 1985) whereas the monofunctional *E. coli* and rat CPSases can form dimers (Trotta et al., 1974; Lusty, 1981).

The loss of CPSase activity parallels the loss of bicarbonate-dependent ATPase, suggesting that inactivation is the result of the modification of the ATP binding site involved in the activation of bicarbonate. Carbamyl phosphate dependent ATP synthesis, the partial reaction thought to be a measure of ATP-driven phosphorylation of carbamate, is also inactivated by 2 mM FSBA but at a rate 3 times slower than the ATPase activity. The difference was even more pronounced at lower FSBA concentrations. At 0.3 mM FSBA, no effect on carbamyl phosphate dependent ATP synthesis was observed when the ATPase was 50% inactivated. These experiments demonstrate that the partial reactions are catalyzed at distinct sites and are consistent with steady-state kinetic studies (Meek et al., 1987) which showed that the high-affinity ATP site (K_m = 0.5 mM cf. 4.2 mM) is associated with the bicarbonate-dependent ATPase activity of CAD.

Controlled proteolysis rapidly cleaves the 154-kDa proteolytic fragment at homologous sites in two halves of the CPS domain. The 140-kDa fragment, which was produced by a

proteolytic cleavage at the carboxyl end of the CPS.B ATP binding region, is in turn cleaved into the 80- and 60-kDa fragments at a trypsin site close to the end of the ATP binding region of CPS.A. When intact CAD was reacted with FSBA, radioactivity was incorporated into both 80- and 60-kDa species, although the extent of labeling of the 60-kDa species is appreciably greater. The observation that both the 80- and 60-kDa proteolytic fragments also react with FSBA at the same rate as the 150- and 140-kDa species indicates that the tertiary structure of these regions of the molecule is preserved even after proteolytic cleavage.

These results, which suggest that the ATP binding regions in both halves of the molecule react with the affinity reagent, were confirmed when FSBA-labeled peptides obtained by exhaustive tryptic digestion were isolated and sequenced. All of the modified peptides mapped within or near the ATP binding regions of CPS.A and CPS.B. The reaction is more specific at low FSBA concentration, and only two peptides are modified. The incorporation of FSBA into peptide T4, located within the 60-kDa fragment, was about 2-fold greater than that into peptide T5 at the amino end of CPS.A (in the 80-kDa fragment). This result is consistent with the observation that the 60-kDa species reacts more rapidly and to a greater extent that the 80-kDa fragment. The modification of 60-kDa species is also reduced to a greater extent if the reaction is carried out in the presence of ATP. Thus T4 is protected somewhat more effectively by ATP than T5. Since ATP protects regions near the amino end of the ATP binding regions of both CPS.A (80 kDa, T5) and CPS.B (60 kDa, T4) under conditions where only one partial reaction is affected, it is likely that reactive residues in both domains are close to the ATPase site. It is interesting that peptide T4 was not modified when the concentration of FSBA was increased 5-fold. Perhaps the conformational change which occurs (M. G. Chaparian, unpublished observations) when ATP binds to the low-affinity site in CAD alters the position of the reactive residue.

The ATP binding domains may each catalyze one of the two ATP-dependent partial reactions (Jones, 1965). Alternately, CPS.A and CPS.B active sites may each catalyze the overall reaction, although this interpretation runs counter to the kinetic scheme (Meek et al., 1987) which suggests that the two ATP binding sites are occupied simultaneously for a time during the catalytic cycle. A third proposal (Carrey & Hardie, 1988) is that one of the ATP binding sites is catalytic, while the nucleotide binding site on the other half of the molecule binds UTP and therefore functions in regulation.

The results reported here provide strong evidence that there are two ATP binding sites, one on each CPSase subdomain, which catalyze different partial reactions. Therefore, both the CPS.A and CPS.B subdomains along with the GLN domain act in concert for the synthesis of carbamyl phosphate. Our results are consistent with the elegant recent studies of Post et al. (1990), who showed that replacement of glycine residues in the amino half of *E. coli* CPSase (analogous to CPS.A) inactivated the bicarbonate-dependent ATPase, while substitutions in a homologous region of the carboxyl half had a greater effect on carbamyl phosphate dependent ATP synthesis.

Although conditions were found which selectively inactivated the partial reaction implicated in bicarbonate activation, peptides close to the ATP binding regions of both CPS.A and CPS.B were modified. Thus we cannot establish which ATP subdomain catalyzes this partial reaction. The close proximity of the ATP binding sites, which has been demonstrated in *E. coli* CPSase in several studies (Powers et al., 1977; Raushel et al., 1983; Kasprzyk et al., 1983), could account for this ambiguity. Considering the structural homology of *E. coli* CPSase and the CPS domain of CAD, it is likely that the two subdomains have homologous functions in both molecules. Once the CPS domain has been cloned and expressed, site-directed mutagenesis studies analogous to those conducted by Post et al. (1990) should allow assignment of functions to the ATP binding sites of CAD.

Acknowledgments

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Synergistic Effect of Histone H1 and Nucleolin on Chromatin Condensation in Mitosis: Role of a Phosphorylated Heteromer

Abelhakim Kharrat,[‡] Jean Derancourt,[§] Marcel Dorée,[§] François Amalric,[‡] and Monique Erard*[,‡]

Centre de Recherche de Biochimie et de Génétique Cellulaires du Centre National de la Recherche Scientifique, 118 Route de Narbonne, 31062 Toulouse Cédex, France, and Centre National de la Recherche Scientifique et Institut National de la Santé et de la Recherche Médicale, B.P. 5051, 34033 Montpellier Cédex, France

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ABSTRACT: Repeated motifs, rich in basic residues, are characteristic of both the N-terminal domain of the nucleolus-specific protein, nucleolin, and the second half of the C-terminal domain of histone H1. These repeats are also the target for phosphorylation by the mitosis-specific $p34^{cdc2}$ kinase. We have previously shown that synthetic peptides [$(KTPKKAKKP)_2$ for histone H1 and $(ATPAKKAA)_2$ for nucleolin] corresponding to these two repeated motifs are able to act in synergy to induce DNA hypercondensation (Erard et al., 1990). In order to determine the molecular basis of this synergistic interaction, we have studied the condensation of the homopolymer poly(dA)·poly(dT) in the presence of the two synthetic peptides. Circular dichroism has been used to monitor the ψ^+-type condensation and has revealed that phosphorylation enhances the synergistic effect of the two peptides. Analysis of different combinations of the two peptides suggests that there is a direct interaction between them which is stabilized by phosphorylation. Furthermore, there is a striking correlation between the degree of homopolymer condensation and the stability of the heteromeric complex. Phosphorylation takes place on the threonine residues on the repeat motifs within a region which is likely to adopt a β-turn structure. Circular dichroism and infrared spectroscopy provide evidence that phosphorylation stabilizes the β-turn structure of both peptides, and computer modeling shows that this may be due to steric hindrance imposed by the phosphate group. We suggest that phosphorylated nucleolin and histone H1 interact through their homologous domain structured in β-spirals in order to condense certain forms of DNA during mitosis.

Although it has been known for some time that histone H1 and its cell cycle dependent phosphorylation is involved in chromatin DNA condensation (Bradbury et al., 1973), the precise nature of the molecular interaction remains to be elucidated. We have recently shown (Erard et al., 1990) that it is the region of the histone H1 C-terminal domain comprising the highly conserved DNA-binding repeat KT(S)-PKKAKKP which performs the function, common to all of the histone H1 proteins, of condensing chromatin linker DNA. Indeed, the H1 repeat can adopt a rigid β-turn-containing structure which binds to the DNA minor groove, dislodging its spine of hydration. The effect on DNA conformation is therefore essentially equivalent to dehydration, which leads

* Corresponding author.
‡ Centre de Recherche de Biochimie et de Génétique Cellulaires du Centre National de la Recherche Scientifique.
§ Centre National de la Recherche Scientifique et Institut National de la Santé et de la Recherche Médicale.

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