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Identification of the Catalytic Subunit of an Oligomeric Casein Kinase (G Type). Affinity Labeling of the Nucleotide Site Using 5′-[*p*-(Fluorosulfonyl)benzoyl]adenosine†

Jean-Jacques Feige, Claude Cochet, Fabienne Pirollet, and Edmond M. Chambaz*

ABSTRACT: Identification of the catalytic subunit of a G type [using guanosine 5′-triphosphate (GTP) as well as adenosine 5′-triphosphate (ATP) as phosphate donor], oligomeric, cyclic nucleotide independent casein kinase purified from bovine lung was carried out after reaction with 5′-[*p*-(fluorosulfonyl)-benzoyl]adenosine (FSBA) and isolation of the subunit components of the enzyme. FSBA exhibited the major characteristics of an affinity label reacting at the nucleotide (ATP, GTP) site of the casein kinase. FSBA acted as a competitive inhibitor of ATP (and GTP), led to complete inactivation of the enzyme in a reaction showing two kinetic steps, and became irreversibly bound to the protein. After being labeled with FSBA, the casein kinase (apparent molecular weight of 140 000) was separated into its two monomeric components of apparent molecular weights 38 000 (α) and 27 000 (β), respectively, after sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Use of radioactive FSBA showed that specific affinity labeling was limited to the α casein kinase subunit. This result was in agreement with the fact that casein kinase activity was found associated with the α monomer after electrophoretic separation of the α and β subunits. It may thus be concluded that the largest (α) subunit contains the catalytic site of the casein kinase G. Electrophoretic analysis of purified protein kinase under denaturing conditions suggested an $\alpha_3\beta_2$ combination for an apparent molecular weight of 130 000–140 000. However, a maximum of 2 mol of FSBA could be specifically bound to the α subunit per mol of enzyme, with a concomitant complete inactivation. These data would be in agreement with an $\alpha_2\beta_2$ subunit composition for casein kinase G, as proposed by other research groups for a similar type of protein kinase of different sources. These observations suggest that the α subunits are functionally similar, each of them containing a nucleotide (ATP, GTP) binding site. The possible role of the β subunit in the enzyme activity remains to be established.

Covalent modification of proteins by phosphorylation–dephosphorylation has emerged as a widely occurring process in the regulation of several important cellular functions (Rubin & Rosen, 1975; Weller, 1979; Cohen, 1981). Various types of ATP (or GTP):protein phosphotransferases (EC 2.7.1.37; protein kinases) have been characterized in mammalian tissues, and criteria for their classification have been proposed (Krebs & Beavo, 1979; Traugh et al., 1974). Whereas messenger dependent protein kinase activities may themselves be regulated by specific intracellular effectors [adenosine cyclic 3′,5′-phosphate (cAMP),[1] cGMP, calcium calmodulin, etc.], protein kinase activities for which no specific effector is yet recognized have been characterized and thus termed messenger independent systems (Krebs & Beavo, 1979). Among this category, cyclic nucleotide independent casein kinases have been classified into two major types: the A type (casein kinase A), using only ATP as phosphate donor, and the G type (casein kinase G), using GTP as well (Cochet et al., 1980). These two types of casein kinases appear similar to the casein kinases I and II (Hathaway & Traugh, 1979) and the casein kinase S (phosphorylating only serine) and TS (phosphorylating both serine and threonine) enzymes (Meggio et al., 1977), respectively. The G-type casein kinase has been purified by several research groups from various tissue sources (Hathaway & Traugh, 1979; Dahmus & Natzle, 1977; Thornburg & Lindell, 1977; Kumon & Ozawa, 1979; Walinder, 1973; Cochet et al., 1981) and usually reported to present an oligomeric structure with an apparent molecular weight between 100 000 and 200 000 for the native enzyme. The G-type casein kinase subunit composition has been reported to be made of two (Walinder, 1973) or three (Hathaway & Traugh, 1979; Dahmus & Natzle, 1977; Kumon & Ozawa, 1979) different moieties, and the smallest subunit is usually recognized as the target of a self-phosphorylation process (Hathaway & Traugh, 1979; Dahmus & Natzle, 1977; Cochet et al., 1981).

However, nothing is known concerning the possible regulation of the cellular G-type casein kinase activity. As a prerequisite, understanding of the molecular organization of the native enzyme and knowledge of the respective roles of the different subunits in the overall catalytic activity may shed some light on the functional properties of this phosphorylation

† From the Laboratoire de Biochimie Endocrinienne, the Institut National de la Santé et de la Recherche Médicale (INSERM), and the Centre National de la Recherche Scientifique (CNRS), Université Scientifique et Médicale de Grenoble, France. *Received August 4, 1982.* This work was supported by the INSERM (U-244, ATP 77.84, and ATP 79.114), the CNRS (ERA 942), and the Fondation pour la Recherche Médicale Française.

* Address correspondence to this author at the Laboratoire de Biochimie Endocrinienne, CERMO, Université Scientifique et Médicale, Grenoble, France.

[1] Abbreviations: FSBA, 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine; ATP, adenosine 5′-triphosphate; GTP, guanosine 5′-triphosphate; Tris, tris(hydroxymethyl)aminomethane; TDG buffer, tris(hydroxymethyl)-aminomethane, dithiothreitol, and glycerol buffer; $NaDodSO_4$, sodium dodecyl sulfate; cAMP, adenosine cyclic 3′,5′-phosphate; cGMP, guanosine cyclic 3′,5′-phosphate.

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system in situ. This study was undertaken with the aim of identifying the catalytic subunit of a purified bovine G-type casein kinase, which exhibits two different subunits under denaturing conditions. The study made use of an ATP analogue, namely, 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine, to affinity label the nucleoside triphosphate site of the enzyme before separation into subunits. Selective labeling of the largest subunit component was observed and was in agreement with the detection of casein kinase activity in the same subunit after isolation under denaturing conditions and partial recovery of the catalytic activity.

Materials and Methods

[γ-^{32}P]ATP (20 Ci/mmol), [γ-^{32}P]GTP (25 Ci/mmol), [2,5′,8-$^{3}H_3$]adenosine (42 Ci/mmol), and NCS gel solubilizer were purchased from the Radiochemical Centre (Amersham); Na^{125}I was from the Commissariat à l'Energie Atomique (Saclay, France). Casein (Merck) was prepared according to Ashby & Walsh (1974) before use. Nucleotides, 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine (FSBA), bovine serum albumin, and spermine were from Sigma. *p*-(Fluorosulfonyl)benzoyl chloride was obtained from Aldrich Chemical Co. Hexamethylphosphoric triamide from Merck-Schuchardt was dried on calcium hydride and redistilled under reduced pressure. Ovalbumin and carbonic anhydrase, used as molecular weight markers, were from Boehringer. AG 1X2 ion-exchange resin was from Bio-Rad Laboratories. TDG medium was 10 mM, pH 7.5 Tris-HCl buffer containing 0.5 mM dithiothreitol and 2% glycerol.

Cyclic nucleotide independent G-type casein kinase was purified from bovine lung cytosol. The method will be described in detail elsewhere. Briefly, it used a combination of phosphocellulose chromatography and affinity adsorption on casein–Sepharose as previously described for the adrenal cortex enzyme (Cochet et al., 1981) with the difference that protease inhibitors were added to the medium before homogenization of the tissue and that an ATP–Sepharose affinity step was included in the procedure. The final preparation appeared homogeneous and showed two bands upon polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate with apparent molecular weights of 38 000 (α) and 27 000 (β), respectively, and an α to β ratio of 2 to 1 upon quantitative scanning after Coomassie blue or amido black staining.

Assay of Enzyme Activity. Protein kinase activity measurements were performed following the incubation conditions described by Corbin & Reimann (1974) by using the trichloroacetic acid precipitation procedure of Sandoval & Cuatrecasas (1976). The standard reaction mixture (80 μL of TDG buffer, pH 7.5) contained 0.1 mM [γ-^{32}P]ATP (specific activity 100 cpm/pmol), 50 mM $MgCl_2$, and 7.5 mg/mL casein. The reaction was initiated by addition of 20 μL of enzymatic solution in TDG buffer containing 2% dimethyl sulfoxide or 10% dimethylformamide and varying amounts of FSBA. [^{32}P]Phosphate incorporation was linear over the 2-min assay period.

Preparation of 5′-[p-(Fluorosulfonyl)benzoyl]adenosine (FSBA). The compound 5′-[*p*-(fluorosulfonyl)benzoyl]-adenosine was synthesized following the procedure of Wyatt & Colman (1977). The product chromatographed together with authentic FSBA on an Eastman thin-layer chromatography cellulose sheet (13254) with fluorescent indicator. The R_f for the compound was 0.88 using 1-butanol–acetic acid–H_2O (5:2:3) as the solvent system. Proton NMR parameters of the final product were identical with those reported in the literature (Di Pietro et al., 1979); the ^{19}F NMR spectrum showed a singlet at 127.66 ppm (with C_6F_6 used as the internal standard) also found with *p*-(fluorosulfonyl)benzoyl chloride.

Radioactive FSBA was prepared from 5 mCi of [2,5′,8-$^{3}H_3$]adenosine (0.1 μmol) added to 0.1 mmol of unlabeled adenosine. Synthesis was performed as described above in the case of unlabeled compound. The specific activity of the resulting isolated [^{3}H]FSBA was 11 000 cpm/nmol. Autoradiography of the corresponding thin-layer chromatogram indicated less than 10% contamination by [^{3}H]adenosine.

Reaction of Radioactive 5′-[p-(Fluorosulfonyl)benzoyl]-adenosine with the Protein Kinase Preparation and Separation of the Labeled Enzyme Subunits. Casein kinase G preparation (70 μg) was incubated (2 h, 30 °C) either with 2 mM [^{3}H]-FSBA or with [^{3}H]FSBA and 1 mM ATP as the protecting agent. Excess [^{3}H]FSBA was removed by two successive precipitations and washings with 90% ethanol. The final 7000*g* pellet was redissolved in 50 μL of electrophoresis buffer containing 10 000 cpm of ^{32}P self-phosphorylated enzyme as the internal standard. The two subunits of the labeled enzyme were then separated by electrophoresis in the presence of 0.1% sodium dodecyl sulfate in a 12% polyacrylamide slab gel, following the procedure of Weber & Osborn (1969). Unlabeled enzyme (15 μg) was run on a parallel lane on the same gel, to be used as a standard after Coomassie blue staining. The sample lanes were sliced into 4-mm-wide sections. Each gel slice was treated with 500 μL of 50% ethanol under continuous shaking in order to extract all free FSBA possibly remaining in the gel; ^{3}H and ^{32}P radioactivity was measured in the ethanol extract at various time intervals thereafter. Whereas a small amount of ^{3}H radioactivity was extracted from the gel within 4 h, ^{32}P remained undetectable in the ethanol extract, thus indicating that this treatment did not remove casein kinase from the gel. Each ethanol-treated slice was then solubilized (2 h at 50 °C) in 1 mL of NCS solubilizer–H_2O (9:1) mixture and counted for ^{3}H and ^{32}P radioactivity in a toluene–Permablend scintillation mixture.

Determination of the Stoichiometry of the Reaction of Casein Kinase with [^{3}H]FSBA. Duplicate experiments were conducted by using 70 μg of enzyme and 2 mM [^{3}H]FSBA dissolved in TDG buffer containing 2% dimethyl sulfoxide at 30 °C for 2 h. The labeled enzyme was precipitated thrice with 90% ethanol, and the final pellet was dissolved in 200 μL of NaOH. Protein concentration was assayed in 20-μL aliquots according to Bradford (1976) with bovine serum albumin as the standard, with the same amount of NaOH as in the assay. The remaining solution was used to measure the incorporated radioactivity after counting in Bray's scintillation fluid.

Catalytic Activity of Casein Kinase G Isolated Subunits. The subunits of casein kinase were separated by polyacrylamide gel electrophoresis in the presence of 0.05% sodium dodecyl sulfate according to Laemmli & Favre (1973). A preelectrophoresis (3 h, 30 °C) was run with 20 μg of ovalbumin on each lane; 6 μg of the casein kinase preparation was then electrophoresed for 4 h at 4 °C. An aliquot of the preparation was radioiodinated by the chloramine T procedure (Hunter & Greenwood, 1962) and run on a parallel lane. The two lanes were washed twice for 20 min in TDG buffer containing AG 1X2 ion-exchange resin and then twice for 20 min in TDG pH 9.0 Tris-HCl buffer containing 0.1 M NaCl and 2% glycerol. The lanes were then sliced into 3-mm-wide sections. The gel slices were either assayed for enzymatic activity or solubilized by NCS solubilizer–H_2O (9:1) and counted for radioactivity in toluene–Permablend scintillation fluid. For the casein kinase activity assay, individual slices were homogenized (Dounce apparatus) in 180 μL of pH 9,

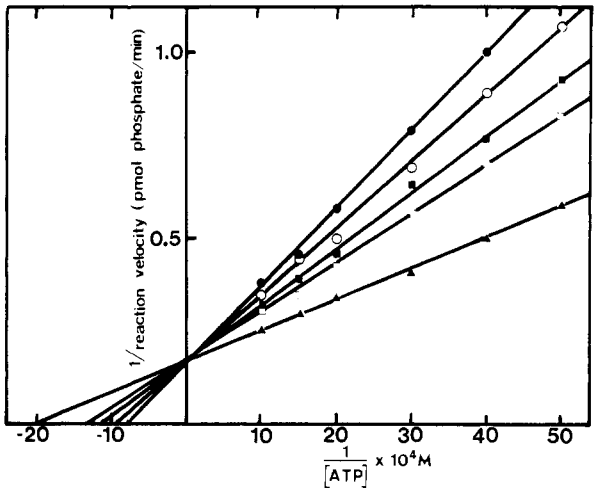

FIGURE 1: Lineweaver–Burk plot of the casein kinase G activity in the presence of FSBA at different concentrations as a function of ATP concentration. Enzyme (0.13 μg of protein/mL) activity was assayed as described under Materials and Methods. Assays were performed at the indicated concentrations of [γ-^{32}P]ATP and FSBA in TDG buffer containing 50 mM $MgCl_2$. Concentrations of FSBA used were as follows: (▲) none; (□) 0.5 mM; (■) 1 mM; (○) 1.5 mM; (●) 2 mM. Velocity is expressed as picomoles of [^{32}P]phosphate transferred from [γ-^{32}P]ATP to casein per minute.

10 mM Tris-HCl buffer containing 1 mg/mL BSA and 6 mg/mL casein and incubated for 4 h at 30 °C in 20 μL of pH 9.0, 10 mM Tris-HCl buffer containing 20 mM spermine, 0.5 M $MgCl_2$, and 5 μCi of [γ-^{32}P]ATP. The incubation mixtures were centrifuged (1000*g*, 10 min); the supernatants were discarded and the acrylamide gel pellets washed twice with 2 mL of 12% boiling trichloroacetic acid. The final pellets were solubilized in 200 μL of NCS solubilizer–H_2O (9:1), and the ^{32}P incorporated into casein was counted in toluene–Permablend scintillation fluid.

Results

Inhibitory Properties of FSBA on Casein Kinase G Activity. The casein kinase activity of the enzyme preparation was assayed in the presence of various concentrations of FSBA and at different concentrations of ATP as the nucleotide substrate. For each set of experiments, the data were plotted according to Lineweaver–Burk. As seen in Figure 1, FSBA acted as a competitive inhibitor of ATP in the casein kinase reaction. Apparent K_m values for ATP at different FSBA concentrations could be calculated from these data. A replot of these K_m values vs. the corresponding FSBA concentrations (Segel, 1975) yielded the linear relationship illustrated in Figure 2. The intercept with the ordinate gave an apparent K_m value for ATP of 5 μM in the absence of FSBA; the intercept with the abscissa yielded an apparent K_i for FSBA toward ATP of about 1.4 mM. These data demonstrated that, although the apparent FSBA affinity for the ATP site of casein kinase G was relatively low as compared to the ATP affinity itself, FSBA acted as a typical competitive inhibitor of the nucleotide at the enzyme level. These observations showed that FSBA may be used with the aim of labeling the ATP (GTP) site in further experiments.

Inactivation of Casein Kinase G upon Incubation with FSBA. The activity of the casein kinase G remained mostly stable after 1 h of incubation at 30 °C in TDG buffer containing 10% dimethylformamide. Addition of various concentrations of FSBA in the medium resulted in a rapid decrease of the enzymatic activity. The ratio of the remaining activity in the presence of FSBA (E) to that of the control (E_0) measured at the same times of incubation was plotted vs. time in a semilogarithmic graph (Ray & Koshland, 1961), as illustrated in Figure 3. This representation showed that the enzyme inactivation at a given time was dependent upon FSBA concentration. Whatever the FSBA concentration, the casein kinase inactivation followed a biphasic rate. After a rapid partial disappearance of the casein kinase activity within 10 min, a slower inactivation process took place within the next 0.5 h. This phenomenon could not be explained by a rapid degradation of FSBA by hydrolysis since electrode-specific measurement of fluorine release indicated a half-life of about 7 h under our incubation conditions. The overall reaction resulted in a practically inert enzyme after 45 min of incubation in the presence of 0.8 mM FSBA. For this reagent concentration, a pseudo-first-order rate constant of $k_1 = 0.43$ min^{-1} for the first step of inactivation could be calculated, whereas a $k_2 = 9 \times 10^{-3}$ min^{-1} was obtained for the second step which is thus about 50 times slower than the first.

When the apparent rate constants k_1 and k_2 calculated for different FSBA concentrations (see Figure 3) were plotted vs. FSBA concentrations, as illustrated in Figure 4, a linear re-

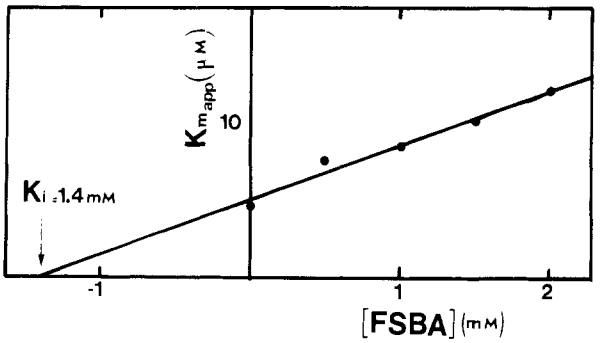

FIGURE 2: Graphical determination of the apparent inhibition constant (K_i) of FSBA relative to ATP for the casein kinase G activity. The apparent K_m values for ATP as determined in the presence of various concentrations of FSBA (graphs given in Figure 1) were plotted vs. the corresponding FSBA concentrations according to Segel (1975).

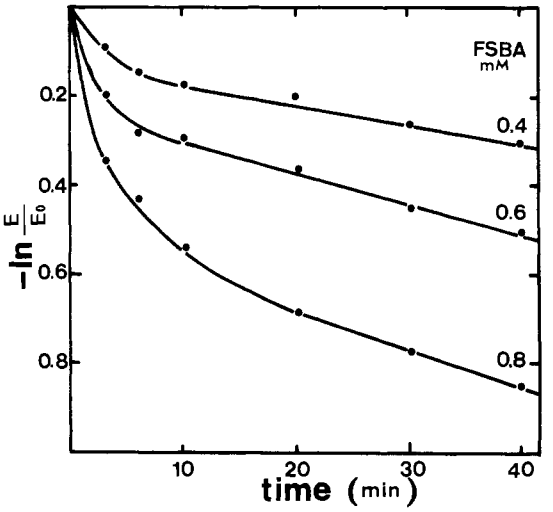

FIGURE 3: Time course of casein kinase G inactivation in the presence of 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine (FSBA). The purified enzyme preparation (3.3 μg of protein) was incubated at 30 °C in TDG buffer containing 10% dimethylformamide and different concentrations (0.4, 0.6, and 0.8 mM) of FSBA, in a final volume of 260 μL. At indicated times thereafter, aliquots (20 μL) of the incubation mixture were withdrawn, and the casein kinase activity was assayed upon 2-min incubation time, as indicated under Materials and Methods. The logarithm of the ratio of the enzyme activity in the presence of FSBA (E) to that of the control value (E_0) in the absence of FSBA, at a given time, was plotted vs. the time of incubation.

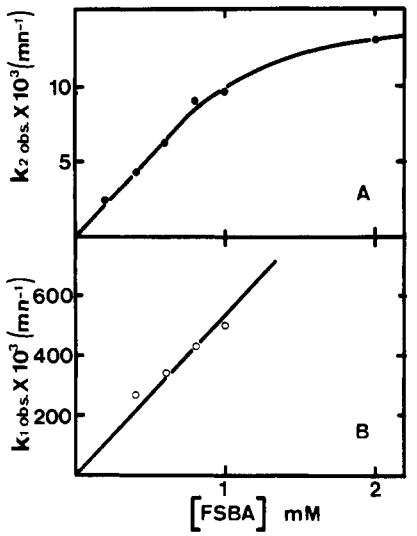

FIGURE 4: Dependence of the pseudo-first-order rate constants of the casein kinase inactivation process by FSBA on FSBA concentration. The pseudo-first-order rates k_1 (panel B) and k_2 (panel A) were determined by a graphical method as indicated under Materials and Methods (Ray & Koshland, 1961) from the data illustrated in Figure 1 for inactivation of casein kinase G at different FSBA concentrations and then plotted vs. FSBA concentrations.

Table I: Effect of Various Nucleotides on the Activity of Casein Kinase G[a]

radioactive nucleotide in the assay	addition	% remaining activity
[γ-^{32}P]ATP	none	100
	ATP	10
	ATPγS	9
	ADP	8
	AMP	92
	adenosine	87
[γ-^{32}P]GTP	none	100
	GTP	24
	GDP	23
	GMP	82
	guanosine	55

[a] Casein kinase G was assayed by using standard assay conditions with 0.1 mM concentration of [γ-^{32}P]ATP or [γ-^{32}P]GTP (1000 cpm/pmol) as substrate and addition of 1 mM concentration of the indicated nucleosides or nucleotides.

lationship was obtained in the case of the faster inactivation process (Figure 4B). By contrast, the pseudo-first-order rate constant of the slower reaction (k_2) reached a plateau when FSBA concentration was increased (Figure 4A) which suggests the occurrence of a saturation phenomenon in the inactivation process.

Casein Kinase G Inactivation by FSBA in the Presence of Nucleotide Substrates or Competitors. Should FSBA be reacting to become covalently bound to the nucleotide catalytic site of casein kinase G in the process of inactivation of the enzyme, one should expect that the presence of the corresponding nucleotide substrates (ATP or GTP) must impair the resulting enzyme inactivation reaction. When the effectiveness of various nucleotides to inhibit casein kinase G reaction supported by ATP (or GTP) was examined (Table I), it was found that nucleoside diphosphates (ADP, GDP) were highly potent in contrast to the corresponding monophosphates (AMP, GMP) and nucleosides. This suggested that nucleoside diphosphates may be used as well as the corresponding triphosphates in competition experiments in the reaction of FSBA with the enzyme. The casein kinase preparation was thus

Table II: Protective Effect of Nucleotides and Magnesium on the Inactivation of Casein Kinase G in the Presence of FSBA[a]

protecting agent	concn (mM)	% inactivation[b]	% protection[c]
none		51	0
$MgCl_2$	10	68	0
ATP	1	22	56
ADP	1	37	28
AMP	1	49	4
GTP	1	29	44
GDP	1	31	40
GMP	1	49	4
ATP + Mg^{2+}	1 + 10	0	100
ADP + Mg^{2+}	1 + 10	31	40
AMP + Mg^{2+}	1 + 10	48	6
GTP + Mg^{2+}	1 + 10	16	68
GDP + Mg^{2+}	1 + 10	27	48
GMP + Mg^{2+}	1 + 10	47	8

[a] Casein kinase preparation (50 ng) was incubated (40 min, 30 °C) in TDG buffer containing 2% dimethyl sulfoxide and 1 mM FSBA, in the absence or in the presence of various combinations of magnesium and nucleotides. At 0- and 40-min times of incubation, aliquots were diluted 50-fold in ice-cold TDG buffer containing 10 mM mercaptoethanol and assayed for casein kinase activity with 0.1 mM [γ-^{32}P]ATP as the phosphate source. [b] (Control activity − activity in the presence of FSBA and protective agent)/control activity. [c] (% inactivation without protective agent − % inactivation with protective agent)/% inactivation without protective agent.

treated with FSBA as described above, after addition of various nucleotides to the medium, and in the absence or presence of magnesium. After the incubation, casein kinase activity was assayed with [γ-^{32}P]ATP as substrate. The results are illustrated in Table II and showed that the presence of ATP or GTP during FSBA treatment resulted in the protection of the enzyme from inactivation. Moreover, the ability of the nucleotides to prevent the inactivation of casein kinase G by FSBA closely paralleled their ability to act as inhibitors in the kinase reaction (see Table I). The nucleoside di- and triphosphates were also more effective when present as their Mg^{2+} complexes. These observations are in agreement with the suggestion that the nucleotide site is indeed the major target of FSBA in the inactivation process. In addition, the effect of FSBA was enhanced when the Mg^{2+} concentration was increased (10 mM), suggesting that an FSBA–Mg^{2+} complex may have a higher affinity for the ATP–GTP site than FSBA alone. This is an additional common property shared by casein kinase G nucleotide substrates (ATP and GTP) and their analogue FSBA.

Evidence for the Irreversible Binding of FSBA to Casein Kinase G. So that it could be established whether the inactivated state of the enzyme was due to an irreversible (covalent) linkage with FSBA, the enzyme preparation was first inactivated to about 80% after incubation with 1 mM FSBA as described above. The incubation mixture was then passed through a Sephadex G-50 minicolumn and the protein eluted under centrifugation as described by Penefsky (1977). Parallel experiments using [^{3}H]FSBA showed that after this gel filtration step, the FSBA concentration dropped from 1 mM (incubation mixture) to 10^{-7} M in the eluate. However, assay of casein kinase activity in the eluate showed that the enzyme remained 80% inhibited with regard to the control. This inactivated state was not modified when the substrate con-

centration (ATP) was increased in the enzyme assay or when the eluted enzyme was preincubated to allow dissociation of possible noncovalently bound FSBA, before assay. It may be recalled that 10^{-7} M FSBA had no significant inhibitory effect when added to the casein kinase assay mixture (see Figure 1). It may thus be concluded that in the FSBA-inactivated enzyme, the nucleotide analogue is apparently associated with the protein through an irreversible linkage. The nucleotide analogue most probably occupies the ATP (GTP) site, which thus becomes completely blocked.

Stoichiometry of the FSBA Reaction with Casein Kinase G. Covalent linkage of the FSBA nucleotidyl residue to casein kinase G was confirmed by quantitative experiments using [^{3}H]FSBA. Following a 120-min incubation at 30 °C of the casein kinase preparation with 2 mM [^{3}H]FSBA (under which conditions the enzyme was totally inactivated), the protein was precipitated 3 times and thoroughly washed with ethanol to remove noncovalently associated reagent. The final precipitate was assayed for protein content and radioactivity incorporation. Two independent experiments yielded values of 3.18 and 3.24 mol of FSBA incorporated per mol of casein kinase, respectively, as calculated on the basis of a 140000 molecular weight for the holoenzyme. It may thus be concluded that, under our experimental conditions, a maximum of 3 mol of FSBA was irreversibly linked to the enzyme.

Identification of the Casein Kinase Subunit Reacting with FSBA. The bovine lung casein kinase preparation used in this work exhibited apparent homogeneity and an apparent molecular weight of 140000 upon gel filtration and sucrose gradient velocity sedimentation analysis. The protein was resolved into two polypeptide bands upon polyacrylamide gel electrophoresis in the presence of $NaDodSO_4$ (Figure 5). The two moieties exhibited M_r's of 38000 (largest component, α) and 27000 (smallest component, β), respectively. Scanning of the electrophoretic trace after Coomassie blue staining and proper correction for the apparent molecular weight indicated a 2:1 ratio between the α and β bands and thus suggested an $\alpha_3\beta_2$ subunit combination in the purified holoenzyme. An identical conclusion was obtained by using a different staining procedure (amido black) or ^{125}I labeling of the holoenzyme (chloramine T procedure) and radioactivity determination in the two isolated enzyme subunits. The casein kinase preparation was analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis after being labeled with [^{3}H]FSBA as described above. As illustrated in Figure 5, measurement of the radioactivity along the corresponding gel trace showed that the α (38K) subunit of the enzyme was coincident with most of the label whereas about one-third of the radioactivity was detected in the gel fractions corresponding to the β (27K) component. When the kinase inactivation by [^{3}H]FSBA was carried out in the presence of 1 mM ATP, a significant decrease of the radioactivity corresponding to the α subunit was observed, whereas labeling of the β-subunit area was not modified. This strongly suggested that β-subunit labeling by FSBA was not related to the presence of a nucleotidic site and was unspecific. It may thus be concluded that the α casein kinase subunit, which was the major target site detected after irreversible labeling with FSBA, contains the nucleotidic site of the enzyme. When it is taken into account that only two-thirds of the enzyme-bound FSBA represented reagent specifically bound to the α subunit, this would mean that only 2 mol of FSBA was incorporated at the nucleotide site per mol of enzyme.

Catalytic Properties of the Isolated α and β Casein Kinase Subunits. A number of unsuccessful attempts were conducted

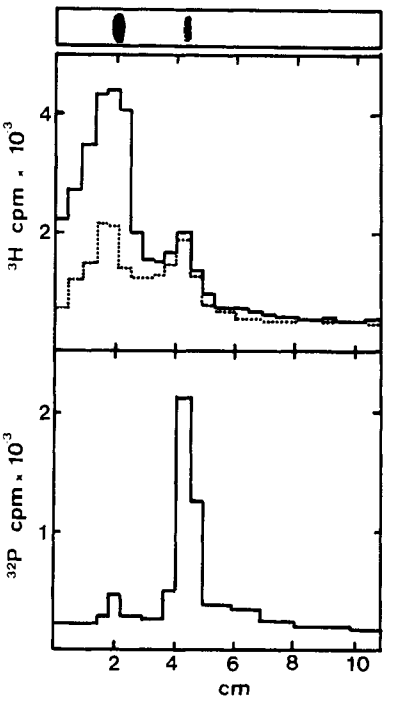

FIGURE 5: Separation of the two casein kinase G subunits after labeling of the purified enzyme with [^{3}H]FSBA and $NaDodSO_4$–polyacrylamide gel electrophoresis. Two samples of the purified casein kinase preparation were reacted with [^{3}H]FSBA in the absence or the presence of 1 mM ATP as protecting agent under the conditions described under Materials and Methods. The affinity-labeled enzyme was found to be 90% inactivated in the absence of ATP and 30% inactivated in the presence of ATP. ^{32}P autophosphorylated enzyme was added as a tracer to both samples before electrophoresis. After the electrophoretic analysis, radioactivity was counted in the sliced gel fractions as described under Materials and Methods. The middle panel represents tritiated radioactivity contained in the slice corresponding to unprotected (solid lines) and ATP-protected (dotted lines) FSBA-inactivated enzyme. The lower panel represents ^{32}P radioactivity contained in the different gel fractions. A parallel lane was run for protein staining by Coomassie blue of an untreated casein kinase sample and is shown in the upper part of the figure. This enabled characterization of the large (α) and small (β) subunits of the casein kinase.

with the aim of finding adequate experimental conditions resulting in the dissociation and separate recovery of the two enzyme subunits. Although dissociation of the two components was obtained in some drastic conditions (e.g., high salt and urea concentrations), it was not possible to recover any enzymatic activity following such treatments. Due to its resolving power (see Figure 5), polyacrylamide gel electrophoresis in the presence of $NaDodSO_4$ was finally employed. Preliminary experiments showed that although native casein kinase G activity was impaired when $NaDodSO_4$ at various concentrations was added to the assay medium, about half of the activity was still detected at 0.05% $NaDodSO_4$ concentration (Table III). In addition, spermine, which has been previously shown to be a potent activator of the G-type casein kinase activity (Cochet et al., 1980, 1981; Job et al., 1979a,b), was found able to somewhat neutralize the deleterious effect of $NaDodSO_4$. As shown in Table III, the casein kinase activation by spermine was higher (×2.8) for the enzyme inhibited at 50% in the presence of $NaDodSO_4$ than for the untreated casein kinase (×1.3).

In a first experiment, casein kinase was electrophoresed in the presence of $NaDodSO_4$ (0.05%) in a polyacrylamide gel (12%) copolymerized with casein (6 mg/mL). Casein kinase activity was then assayed in the gel after slicing, using casein included in the gel as the protein substrate and addition of

Table III: Protective Effect of a Polyamine (Spermine) upon Casein Kinase G Inactivation by Sodium Dodecyl Sulfate[a]

$NaDodSO_4$ concn (%)	casein kinase activity[b]		activation factor elicited by spermine
	no spermine	1 mM spermine	
0	100	130	1.3
0.005	95	ND	ND
0.05	50	85	2.8
0.5	10	ND	ND
5	1	ND	ND

[a] Casein kinase activity was assayed in TDG buffer containing various concentrations of $NaDodSO_4$, in the absence or in the presence of 1 mM spermine. [b] The enzymatic activity was expressed as percent relative to the control (obtained in the absence of $NaDodSO_4$ and spermine), taken as 100%. ND, not determined.

[γ-^{32}P]ATP. No casein kinase activity could be detected under these conditions, even when the gel slices were thoroughly treated with AG 1X2 resin and spermine was added to the assay medium.

A second set of experiments was carried out where the α and β casein kinase subunits were first separated by $NaDodSO_4$ (0.05%)–polyacrylamide gel (12%) electrophoresis. The gel was then repeatedly washed in the presence of AG 1X2 resin and bovine serum albumin. After being sliced, the gel was homogenized and each slice incubated with casein and [γ-^{32}P]ATP in the presence of spermine for casein kinase assay as described under Materials and Methods. The reaction mixture was then treated with 12% boiling trichloroacetic acid and the radioactivity measured in the centrifugation pellet. As shown in Figure 6, a casein kinase activity could be detected under these conditions and was found only in the gel slices containing the α subunit of the enzyme. Identical results were obtained when GTP was used as the substrate instead of ATP. It may thus be concluded that the α subunit exhibits casein kinase G activity. However, since protein recovery evaluation was not possible in these experiments, it cannot be ascertained whether separation from the β subunit resulted in any change in the α-subunit casein kinase specific activity.

Discussion

The aim of this work was at first to get some information concerning the molecular organization of an oligomeric messenger-independent protein kinase isolated from bovine tissue (casein kinase G) and particularly to identify the catalytic subunit of the enzyme. Bovine casein kinase G purified to apparent homogeneity (apparent M_r 140 000) is dissociated into α (M_r 38K) and β (M_r 27K) subunits in the presence of sodium dodecyl sulfate, and quantitative scanning suggested an $\alpha_3\beta_2$ combination for the native form of the enzyme. Casein kinase G appears similar to casein kinase II (Hathaway & Traugh, 1979; Dahmus, 1981), casein kinase TS (Meggio et al., 1977), glycogen synthase kinase PC 0.7 (DePaoli-Roach et al., 1981), and GSK-5 (Cohen et al., 1982) described by others. However, the oligomeric structure has been reported to be made of three (Dahmus, 1981) or two subunits (Hathaway et al., 1981; Huang et al., 1982, DePaoli-Roach et al., 1981). Since the G-type casein kinase belongs to the category of messenger-independent protein kinases (Krebs & Beavo, 1979), knowledge of the respective role of the two subunits may contribute to the understanding of the possible regulation of the enzyme in situ.

In this study, identification of the casein kinase subunit bearing the catalytic site was carried out by affinity labeling

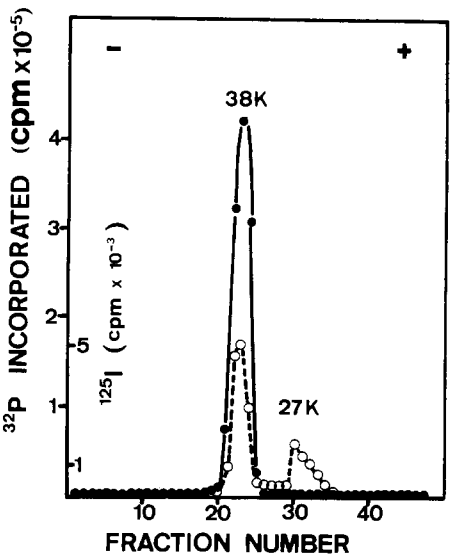

FIGURE 6: Demonstration of casein kinase activity comigrating with the α subunit of casein kinase G after $NaDodSO_4$–polyacrylamide gel electrophoresis. Purified casein kinase G preparation was analyzed by polyacrylamide (12%) gel electrophoresis under denaturing conditions, in the presence of $NaDodSO_4$ (0.05%). After being washed with an ion-exchange resin, a bovine serum albumin solution, and TDG buffer, casein kinase activity (●) was then assayed along the gel lane, as described under Materials and Methods. ^{125}I-Labeled casein kinase G was analyzed on a parallel lane of the same gel in order to increase the sensitivity of the detection of the α (38K) and β (27K) subunits of the enzyme (○).

using a reactive ATP analogue, namely, 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine (FSBA), which was also synthesized in ^{3}H-labeled form. This nucleotide analogue has been previously used by others to affinity label the nucleotide site of several dehydrogenases (Pal et al., 1975; Roy & Colman, 1979) and the ATP site of pyruvate kinase (Wyatt & Colman, 1977), mitochondrial ATPase (Di Pietro et al., 1979; Esch & Allison, 1978), carbamoyl-phosphate synthetase (Boettcher & Meister, 1980), luciferase (Lee et al., 1981), and recently the cyclic AMP dependent protein kinase catalytic subunit (Hixson & Krebs, 1979; Zoller & Taylor, 1979) and the cyclic GMP dependent protein kinase (Hixson & Krebs, 1981). When this work was in progress, a report was published describing the use of FSBA to characterize the catalytic site of the erythrocyte casein kinase II (Hathaway et al., 1981). Casein kinase II appears highly similar to the casein kinase G and presents a two-subunit structure, with α (43K) and β (26K) components with an $\alpha_2\beta_2$ stoichiometry. FSBA labeling of casein kinase II was selective of the α subunit with inactivation kinetics similar to those observed in this study.

FSBA was thought to be adaptive to the aim of the present work since the casein kinase G catalytic site accepts both ATP and GTP and since the reactive end of FSBA has a location similar to that of the γ-phosphoryl group of ATP when the molecule is in an extended conformation (Wyatt & Colman, 1977). The reaction of casein kinase G with FSBA was found to exhibit the major characteristics expected for an affinity labeling process; FSBA was found to inactivate casein kinase G in a concentration-dependent and a time-dependent manner, the inactivation process being impaired by the nucleotide substrates (ATP and GTP) of the enzyme. Kinetic studies showed two steps in the inactivation reaction. Whereas a rapid inactivation reaction exhibited an apparent rate constant linearly related to FSBA concentration, a slower inactivation process showed a saturation phenomenon in the rate constant–FSBA concentration relationship. These findings are very similar to the observations of Wyatt & Colman (1977)

in the case of pyruvate kinase and that of Di Pietro et al. (1979) with a mitochondrial ATPase. These workers interpreted their data as indicating a reversible binding of FSBA to the nucleotide site, prior to irreversible covalent reaction, and suggested two different loci on the protein as being able to associate with FSBA. It may be of interest in our case to evaluate the relative amount of FSBA reversibly associated or irreversibly bound to the enzyme at a given time, throughout the FSBA–casein kinase reaction process. However, a possible regulatory site able to reversibly bind ATP (and therefore FSBA) has not been demonstrated in the case of casein kinase G.

The α subunit of casein kinase G appeared to be the only component bearing a nucleotidic site representing a specific target for FSBA covalent linking. This is in agreement with the observation that this subunit was the only component of the enzyme exhibiting casein kinase activity, after isolation from $NaDodSO_4$–polyacrylamide electrophoretic gels. Since only 2 mol of FSBA could be specifically bound to the α subunit per mol of native enzyme, an $\alpha_2\beta_2$ stoichiometry is suggested and would fit with a 130 000–140 000 apparent molecular weight as determined by gel filtration. Taken together, the observations made in this study suggest that the most likely casein kinase G structure is an $\alpha_2\beta_2$ subunit combination in which the α components are equivalent, each of them bearing one of the catalytic sites of the enzyme. On the other hand, it remains to be established whether the 27K, β subunit plays any role in the casein kinase activity of the α monomer in the native multimeric enzyme. Work is in progress in the laboratory to examine this point by an approach using isolated α and β monomers and recombination experiments. On the other hand, it may be pointed out that the β monomer is rapidly self-phosphorylated when native casein kinase is incubated with ATP (or GTP) (Hathaway & Traugh, 1979; Dahmus & Natzle, 1977; Thornburg & Lindell, 1977; Kumon & Ozawa, 1979; Walinder, 1973; C. Cochet et al., unpublished results). This may suggest that the 27K component could be an endogenous substrate, which remained tightly associated with the enzyme throughout the purification procedure. However, this is not likely since the α:β ratio remained unchanged after an additional adsorption–desorption cycle of the purified enzyme on phosphocellulose. In addition, the enzyme preparation was strongly adsorbed on affinity systems prepared with a protein substrate such as casein–Sepharose, which suggests that the casein site of the enzyme is not likely to be blocked by the presence of an endogenous substrate. When the 38K and the 27K casein kinase components are available, this will also provide the possibility of defining the site(s) concerned with the interaction of the enzyme with previously described effectors of the native casein kinase activity. Of special interest would be the study of the target site of an endogenous specific inhibitor of the enzyme isolated from bovine tissues (Job et al., 1979a,b), since this inhibitor represents a potential modulator of casein kinase G activity in situ. On the other hand, polyamines such as spermine have been found to be potent activators of casein kinase G activity (Cochet et al., 1980, 1981; Job et al., 1979a,b). These compounds may be of significance for the intracellular regulation of the G-type casein kinase activity. Therefore, study of their interaction with the monomeric components of casein kinase G may shed some light on the regulatory processes concerning the native enzyme in situ.

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Registry No. FSBA, 57454-44-1; ATP, 56-65-5; ADP, 58-64-0; GTP, 86-01-1; GDP, 146-91-8; ATP·Mg, 1476-84-2; ADP·Mg, 7384-99-8; GTP·Mg, 28141-84-6; GDP·Mg, 7277-99-8; spermine, 71-44-3; casein kinase, 52660-18-1.

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Amino Acid Sequence of Crayfish (*Astacus fluviatilis*) Trypsin I_f[†]

Koiti Titani, Tatsuru Sasagawa, Richard G. Woodbury, Lowell H. Ericsson, Herbert Dörsam, Marinette Kraemer, Hans Neurath, and Robert Zwilling*

ABSTRACT: The complete amino acid sequence of trypsin from the crayfish *Astacus fluviatilis* has been determined. The protein was fragmented with cyanogen bromide after S-carboxymethylation of the reduced disulfide bonds and by trypsin after S-carboxymethylation as well as after succinylation of lysine residues and aminoethylation of the reduced disulfide bonds. Peptides were purified by gel filtration and by reversed-phase high-performance liquid chromatography. Stepwise degradation was performed in a spinning cup sequencer. The enzyme contains 237 amino acid residues and has a molecular weight of 25 030. In contrast to bovine trypsin, it contains three rather than six disulfide bonds which are paired in the same fashion as those in trypsin from *Streptomyces griseus*. The constituents of the active site of bovine trypsin are present in corresponding positions in the crayfish enzyme. Crayfish trypsin shows 43.6% sequence identity with the bovine enzyme as compared to 40.0% identity with the *S. griseus* enzyme. The present analysis affords the first detailed view into the evolution of trypsins at the invertebrate level.

Ever since the isolation of crystalline trypsin by Kunitz & Northrop (1936), this enzyme has served as a prototype in the study of the structure and function of proteolytic enzymes. Trypsin-related proteases fulfill a variety of physiological functions (Reich et al., 1975; de Haën et al., 1975), and their phylogeny has been traced back to the prokaryotic level (Olafson et al., 1975).

Despite the current detailed knowledge of the structure and function of mammalian serine proteases, of which trypsin is a prominent member, information of the early evolutionary history of this family of proteases is relatively fragmentary. Indeed, it is remarkable how much less is known about the invertebrate proteases than about their vertebrate or bacterial counterparts (Zwilling & Neurath, 1981). In order to fill this gap of the phylogenetic development of trypsins, and identify those amino acid residues which have been conserved during the evolution of the enzyme, we have undertaken a determination of the amino acid sequence of crayfish trypsin. This "old" serine protease lies on the evolutionary pathway from which decapode crustacea and mammals have diverged some 700 million years ago.

In contrast to its mammalian counterpart, crayfish trypsin is a rather acidic protein. It is resistant to autodigestion, is irreversibly denatured below pH 3, and has no known zymogen form (Zwilling et al., 1969). Some of its structural features are more closely akin to bacterial than to bovine trypsin (Zwilling et al., 1975). The present study describes the complete covalent structure of this invertebrate trypsin which, for the reasons given above, might be considered a "missing link" between prokaryotic and vertebrate serine proteases (Zwilling et al., 1980).

Materials and Methods

Crayfish trypsin is elaborated by the hepatopancreas and is secreted into the stomachlike cardia, which is the best source for this enzyme. The dark brown digestive fluid (containing approximately 3 mg of trypsin/mL) was collected from the cardia by the method previously described (Zwilling & Neurath, 1981). Initially, the enzyme was purified by affinity chromatography on soybean trypsin inhibitor (SBTI)[1] covalently linked to Sepharose (Zwilling & Neurath, 1981). This procedure provided a highly purified preparation in good yield but failed to separate the five multiple forms known to coexist in trypsin of *Astacus fluviatilis* (Pfleiderer & Zwilling, 1972). In order to effect their separation, we subjected the crude extract to anion-exchange chromatography on DEAE-Sephacel (pH 8.0, NaCl gradient, 0.2–2.2 M) and to gel filtration on Sephadex G50, fine (3 × 95 cm column, 0.01 M Tris-HCl buffer, pH 8.0, containing 0.4 M NaCl), repeating each step

[†] From the Howard Hughes Medical Institute and the Department of Biochemistry, University of Washington, Seattle, Washington 98195 (K.T., T.S., R.G.W., L.H.E., and H.N.), and the Department of Physiology, Institute of Zoology, University of Heidelberg, D-6900 Heidelberg, FRG (H.D., M.K., and R.Z.). *Received October 13, 1982.* This work was supported by the National Institutes of Health (GM-15731) and by research grants from the Deutsche Forschungsgemeinschaft, Bonn-Bad Godesberg, FRG (Zw 17/5 and Zw 17/7).

[1] Abbreviations: I_f, band I of crayfish (*Astacus fluviatilis*) trypsin; AE, aminoethyl; CM, carboxymethyl; HPLC, high-performance liquid chromatography; Pth, phenylthiohydantoin; TPCK, tosylphenylalanyl chloromethyl ketone; SBTI, soybean trypsin inhibitor; DEAE, diethylaminoethyl; Tris, tris(hydroxymethyl)aminomethane.

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