Sekimizu, K., Kobayashi, N., Mizuno, D., and Natori, S. (1976), *Biochemistry 15,* 5064.
Sekimizu, K., Mizuno, D., and Natori, S. (1977), *Biochim. Biophys. Acta* (in press).
Shea, M., and Kleinsmith, L. J. (1973), *Biochem. Biophys. Res. Commun. 50,* 473.
Stein, H., and Hausen, P. (1970), *Cold Spring Harbor Symp. Quant. Biol. 35,* 473.
Stewart, L. E., and Krueger, R. C. (1976), *Biochim. Biophys. Acta 425,* 322.
Sugden, B., and Keller, W. (1973), *J. Biol. Chem. 248,* 3777.

Adenosine 3′:5′-Monophosphate Dependent Protein Kinase from Bovine Heart. Characterization of the Catalytic Subunit†

Kristine A. Peters, Jacques G. Demaille,‡ and Edmond H. Fischer*

ABSTRACT: The catalytic subunit of adenosine 3′:5′-monophosphate dependent protein kinase has been isolated in pure form from bovine heart with a 37% yield. The monomeric species (mol wt 39 000) exhibits a strong tendency to aggregate at neutral pH and low ionic strength. It contains 7 tryptophans ($A_{280,1cm}^{1\%}$ = 14.9), no carbohydrate, and 1.7 equiv of acid-stable protein-bound phosphate. The amino terminus of the polypeptide chain is blocked. By isoelectric focusing, 3 major isozymes (pI = 7.01, 7.48, and 7.78) can be separated; they exhibit a similar phosphate content but might differ in their amino acid composition. The catalytic subunit contains three sulfhydryl groups which can be titrated by 5,5′-dithiobis(2-nitrobenzoic acid) with $t_{1/2}$ = 0.36, 3.5, and 110 min, respectively. Substitution of the most reactive thiol group does not inactivate the enzyme; inhibition results from the titration of the second group (presumably located close to the active site) and the third (buried) SH group. None of the cysteinyl side chains is directly involved in catalysis since the percyanylated protein retains 63% of the activity of the native enzyme. The percyano derivative is immune to iodoacetamide inactivation and exhibits an intact protein–substrate binding site, as probed by the use of the protein kinase inhibitor. Cleavage of the *S*-cyano catalytic subunit at pH 9.0 yields a fragment of mol wt 23 000, that originates from the amino end of the molecule, plus two overlapping peptides of mol wt 16 500 and 15 000 that appear to be located on the COOH-terminal half of the protein.

The effects of the cyclic nucleotides, produced upon stimulation of the corresponding cyclases, are mediated through the activation of protein kinases (ATP:protein phosphotransferases, EC 2.7.1.37) which transfer phosphoryl groups to seryl or threonyl side chains of various protein substrates (for reviews, see Walsh and Krebs, 1973; Rubin and Rosen, 1975).

Whereas the cGMP[1]-dependent protein kinase is activated without dissociation into lower molecular weight subunits (Gill et al., 1976; Lincoln et al., 1977), the cAMP-dependent protein kinases, upon binding of the nucleotide, dissociate into two catalytically active subunits (C) and a dimeric, cAMP-regulatory subunit complex (Hofmann et al., 1975).

It is thus possible to study separately the catalytic and the regulatory moieties of the molecule on both a structural and functional level. As a first step toward elucidating the primary structure of the catalytic subunit from bovine heart cAMP-dependent protein kinase (peak II), the enzyme has been isolated in pure form. Some of its properties were studied, with special reference to the several components obtained upon isoelectric focusing, and the reactivity of its thiol groups. Bovine heart was chosen because it is readily available; it contains a high concentration of the cAMP-dependent protein kinase (Hofmann et al., 1977) on which a considerable amount of information is already available (Rosen and Erlichman, 1975; Hofmann et al., 1975; Rangel-Aldao and Rosen, 1976a,b).

Materials and Methods

Histone IIA and protein markers were obtained from Sigma; ovalbumin and pepsin (3100 U/mg) were from Worthington. $^{32}P_i$, [^{14}C]KCN, and 2,5-diphenyloxazole were from New England Nuclear. 5,5′-Dithiobis(2-nitrobenzoic acid) (Nbs_2), citraconic anhydride, and all reagents used in the sequencer were from Pierce. [γ-^{32}P]ATP, ca. 780 mCi/mmol, was prepared by the procedure of Glynn and Chappell (1964). The protein inhibitor of cAMP-dependent protein kinases (PKI) was isolated from rabbit skeletal muscle as previously described (Demaille et al., 1977). The assays for protein kinase and for PKI were also carried out according to Damaille et al. (1977) except that dithiothreitol or 2-mercaptoethanol was omitted from the incubation mixture unless otherwise specified.

Isolation of the Catalytic Subunit. The catalytic subunit of the cAMP-dependent protein kinase (peak II) was prepared from bovine heart essentially as previously described (Beavo et al., 1974; Bechtel et al., 1977; Demaille et al., 1977) with the following minor modifications. (a) C subunit solutions were

† From the Department of Biochemistry, University of Washington, Seattle, Washington 98195. *Received August 11, 1977.* This work was supported by grants from the National Institutes of Health, Public Health Service (AM 07902), the National Science Foundation (GB 3249), and the Muscular Dystrophy Association, Inc.

‡ On leave of absence from the Medical School, University of Montpellier I. Present address: CNRS, 34033 Montpellier, Cedex, France.

[1] Abbreviations used are: C subunit, the catalytic subunit of the adenosine 3′:5′-monophosphate dependent protein kinase; PKI, protein kinase inhibitor; cAMP, cyclic adenosine 3′:5′-monophosphate; cGMP, cyclic guanosine 3′:5′-monophosphate; EDTA, ethylenediaminetetraacetic acid; CM, carboxymethyl; Tris, tris(hydroxymethyl)aminomethane; UV, ultraviolet; P_i, inorganic phosphate; Gdn·HCl, guanidine hydrochloride; Nbs_2, 5,5′-dithiobis(2-nitrobenzoic acid); Nbs, nitrothiobenzoate; Pth, phenylthiohydantoin.

concentrated on Amicon PM30 membranes, which retained more than 95% of the enzyme. (b) Successive adsorptions on CM-Sephadex C-50 were all carried out at pH 6.1 in order to increase the yield. This also resulted in a greater amount of high molecular weight contaminants, which were easily separated at the last gel filtration step. (c) Instead of the affinity chromatography on Cibacron-blue-Sepharose, Sephacryl S-200 superfine (Pharmacia AB, Uppsala) was used. This resulted in higher yields with an equal degree of purification.

Isoelectric focusing was performed in the 440-mL LKB apparatus using equal amounts of 1% Ampholine pH 5–8 and pH 7–10. This provided a shallow pH gradient between 7 and 8 where the C subunit was expected to focus. Cathode was at the bottom of the gradient. Ampholytes were allowed to prefocus overnight at 2 °C, 400 V. Ten milliliters was withdrawn from the middle of the sucrose gradient (d = 1.13) and added to 3 mL of a C subunit solution in 30 mM potassium phosphate, 0.1 mM EDTA, 15 mM 2-mercaptoethanol, pH 7.0. Density was readjusted to 1.13 by addition of solid sucrose, and the mixture was reinjected into the column at the same level. Focusing was carried out for an additional 26 h under 600 V. The column was then emptied and each fraction was assayed for absorbance at 280 nm, pH, and kinase activity.

Gel Electrophoresis. Molecular weight was determined by electrophoresis in 0.1% dodecyl sulfate–15% polyacrylamide slab gels, using the discontinuous Tris–glycine buffer system of Laemmli (1970) with ovalbumin (43 000), carbonic anhydrase (29 000), β-lactoglobulin (17 500), and lysozyme (14 300) as markers. Polyacrylamide (7.5%) disc gel electrophoresis was carried out at pH 8.9 in the absence of denaturing agents, according to Pechère et al. (1971); 7.5% polyacrylamide slab gel electrophoresis at pH 4.3 was carried out in the buffer system of Reisfeld et al. (1962), with the following modifications: separating and stacking gels contained 8 M urea; the sample was dissolved in 8 M urea, 3.12% β-alanine, 0.025% methyl green buffer adjusted to pH 6.8 with acetic acid.

Gels were stained for proteins with Coomassie brilliant blue R, and for carbohydrates with the periodic acid–Schiff reagent (Glossmann and Neville, 1971). When the samples were labeled with ^{14}C, gels were soaked in 2,5-diphenyloxazole (22.2% w/v in dimethyl sulfoxide) before desiccation (Bonner and Laskey, 1974); fluorography was then obtained after 24-h exposure of an x-ray film (Kodak RP X-Omat). Densitometry of stained gels and their fluorograms was carried out with a "Quick Scan" Densitometer, Helena Lab., Beaumont, Tex., using the 610 and 570 filters, respectively.

Analytical Techniques. Protein concentration was measured according to Lowry et al. (1951). Concentration of pure C subunit was determined spectrophotometrically using an absorbancy index $A_{280,1\ cm}^{1\%}$ = 14.9 (see Results).

Amino acid analyses were performed in a Durrum analyzer (Model D500) according to Moore and Stein (1963), after 24, 48, and 72 h of hydrolysis in 5.7 N HCl at 110 °C. Cysteine was determined as cysteic acid (Hirs, 1967) and tryptophan according to Hugli and Moore (1972). The NH_2 terminus of the molecule was studied in the sequencer as described by Hermodson et al. (1972).

Acid-stable, protein-bound phosphate was measured on 5–10 nmol of protein precipitated twice by 30% trichloroacetic acid from a maximum volume of 0.2 mL and then incubated in 0.2 mL of 16% trichloroacetic acid at 90 °C for 20 min. The final pellet was redissolved in 88% formic acid. One-half of the solution was used for protein determination, the other was ashed according to Ames (1966) in parallel with potassium phosphate standards. Phosphate was then measured in triplicate by the malachite green method of Itaya and Michio (1966). Acid-washed plastic and glassware were used throughout.

Kinetic Study of Sulfhydryl Group Modification. Thiol-free solutions of the C subunit were obtained by passing 1-mL aliquots of a 4.8 mg/mL solution of the enzyme through a 0.9 × 50 cm column of Sephadex G-25 equilibrated with 30 mM potassium phosphate, 1 mM EDTA, 0.15 M KCl, pH 7.0. Reaction of the native protein with thiol reagents was stopped at appropriate times by a 150-fold dilution to ca. 5–10 μg/mL with the ice-cold phosphate–EDTA–KCl buffer described above. This dilution also afforded an appropriate level of activity in the enzyme assay. Reaction of the native enzyme with Nbs_2 was followed in a Cary Model 15 recording spectrophotometer using a molar absorbancy $\epsilon_{412\ nm}$ = 13 600 M^{-1} cm^{-1} for the nitrothiobenzoate anion (Ellman, 1959). In the calculation of the rate constants, corrections were made to account for the contribution of the slower reacting groups.

Total SH groups were determined by carrying out the Nbs_2 reaction in the presence of 6 M Gdn·HCl (or 2% dodecyl sulfate), 80 mM sodium phosphate, 2 mM EDTA, pH 8.0 (Habeeb, 1972).

Preparation of S-Cyano C Subunit. *S*-Cyano C subunit could be prepared equally well by reacting the protein with 2-nitro-5-thiocyanobenzoic acid (Jacobson et al., 1973; Degani and Patchornik, 1974) or by the two-step procedure of Vanaman and Stark (1970). In the latter instance, the C subunit (10–20 μM in the pH 7.0 phosphate–EDTA–KCl buffer, described above) was first reacted with a tenfold excess of Nbs_2 and the reaction was followed at 412 nm until full substitution of the thiol groups. Excess reagent was removed by gel filtration on Sephadex G-25; after addition of 50 mM KCN, the pH was immediately readjusted to ≤8 and the reaction was allowed to proceed at 0 °C for 18 h. Excess reagents were separated by gel filtration on Sephadex G-25 in the pH 7.0 buffer.

S-[^{14}C]Cyano C subunit was prepared by the same procedure using undiluted [^{14}C]KCN (9 mCi/mmol). Protein-bound cyanide was measured, before and after thiolysis, by counting 100-μL aliquots of the Sephadex G-25 excluded peak in 10 mL of scintillant (8 g of Omnifluor and 125 g of naphthalene per L of dioxane).

Cleavage of the *S*-cyano C subunit could not be performed in 6 M Gdn·HCl solution (Jacobson et al., 1973), because the peptide fragments generated were insoluble even in 1% dodecyl sulfate after desalting and lyophilization. The best yield was obtained when *S*-cyano C-subunit (60 μM) was cleaved at 37 °C for 18 h in 1% dodecyl sulfate, 0.2 M sodium borate, pH 9.0. The cleavage products were readily separated in a thick (2.5 mm) 0.1% dodecyl sulfate–15% polyacrylamide gel (6 h migration at 36 mA). After extensive washing with the usual methanol–acetic acid–water solutions to remove free glycine, bands were cut out from the gel and homogenized in a 1% dodecyl sulfate, 0.2 M *N*-ethylmorpholine–acetic acid buffer, pH 7.5. Acrylamide particles were removed by centrifugation, then by filtration through a 0.45-μm Millipore filter. The clear solution was dialyzed and lyophilized prior to amino acid analysis.

Alternatively, cleavage fragments were citraconylated (Atassi and Habeeb, 1972) and then separated on a 1.5 × 164 cm column of Sephadex G-150 equilibrated with 0.1% dodecyl sulfate, 50 mM sodium borate buffer, pH 9.0, and eluted at 4 mL/h. Each fraction was analyzed by 0.1% dodecyl sulfate–15% polyacrylamide slab gel electrophoresis and the pure peptides were dialyzed and lyophilized prior to amino acid analysis.

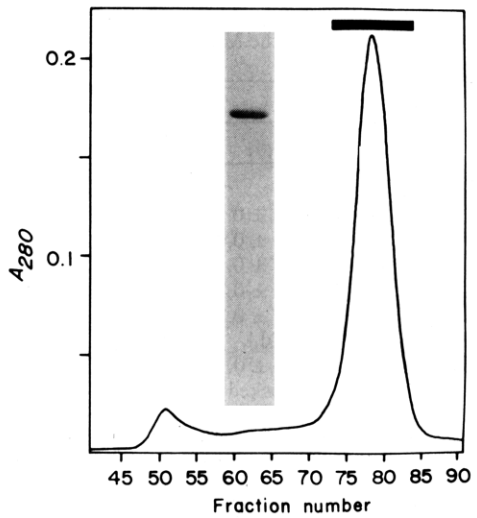

FIGURE 1: Elution profile of the C subunit from Sephacryl S-200. After salt elution from CM-Sephadex C-50 (see Materials and Methods), the protein was pooled and concentrated to 20 mL. Insoluble material was removed by centrifugation (13 000*g*, 15 min) and the supernatant passed at 16 mL/h, 4 °C, through a 2.6 × 95 cm column of Sephacryl S-200 in 30 mM potassium phosphate, 0.1 mM EDTA, 0.15 M KCl, 1 mM dithiothreitol, pH 6.7. Absorbance at 280 nm was recorded and fractions of 4 mL were collected. Fractions containing the C subunit were pooled as indicated, concentrated over Amico PM 30 to 4.8 mg/mL, and stored at 0 °C. C subunit (2.4 μg) was analyzed by 0.1% dodecyl sulfate–15% polyacrylamide gel electrophoresis (see Materials and Methods). Relative migration to the dye front = 0.19.

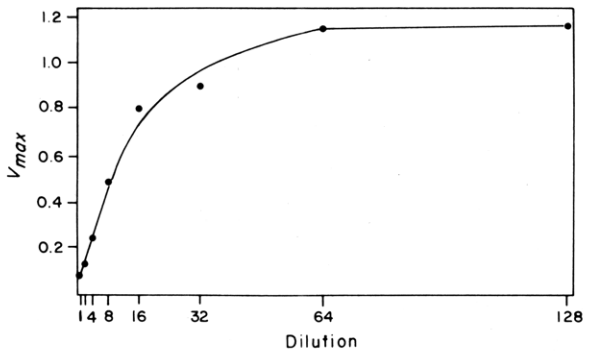

FIGURE 2: Increase in specific activity of the catalytic subunit upon dilution. A solution of C subunit (210 nM or 8.4 μg/mL) was serially diluted and 20 μL of each dilution assayed in a final volume of 70 μL. Plateau is reached at V_{max} = 1.16 μmol min^{-1} mg^{-1} at 30 °C. At the highest dilution ($^1/_{128}$), incorporation of ^{32}P into mixed histones is still ca. 3 times higher than in the blank.

Results

Preparation, Stability, and Storage of the Catalytic Subunit. The catalytic subunit of protein kinase was purified to homogeneity with a ca. 37% yield (5.5 mg/kg of myocardium), assuming an initial concentration of 0.38 μmol/kg wet wt (Hofmann et al., 1977). The enzyme is homogeneous in 0.1% dodecyl sulfate–15% polyacrylamide gel (Figure 1) and migrates with a mol wt of 39 000; it is eluted from the Sephacryl S-200 column with a V_e/V_0 = 1.53 (K_{av} = 0.36) corresponding to the monomer, in good agreement with the mol wt value of 38 000 previously reported for the native subunit by Erlichman et al. (1973). Homogeneous C subunit exhibits a considerable increase in specific activity upon dilution (Figure 2); a similar activation by dilution was already reported by Hofmann et al. (1977) in crude extracts, where it was suggested that inhibitory factors might be diluted out. Of course, this cannot be the case for the pure C subunit. An alternative explanation is that the enzyme has a strong tendency to aggregate at neutral pH below a certain ionic strength. In fact, whereas the material eluted from Sephacryl S-200 with a buffer containing 0.15 M KCl showed no evidence for aggregation, dialysis of the enzyme vs. a neutral low ionic strength buffer usually led to a heavy precipitate and significant losses in activity. Concentrated neutral solutions of C subunit also form a filamentous precipitate at the glass–water interface. Another striking feature is that the enzyme does not migrate into 7.5% polyacrylamide gels in the absence of denaturing agents, both at pH 8.9 and 4.3. In 8 M urea, pH 4.3, most of the enzyme remains at the interface between stacking and separating gels; the small fraction of enzyme which migrates into the gel exhibits a mobility of 0.24 relative to methyl green. When the protein load is increased above 20 μg, several intermediate bands become visible with a characteristic logarithmic spacing corresponding to multiples of the monomeric species (not illustrated). The best storage condition to avoid aggregation is 3–5 mg of protein/mL in a neutral buffer of ionic strength >0.15, containing 1 mM dithiothreitol.

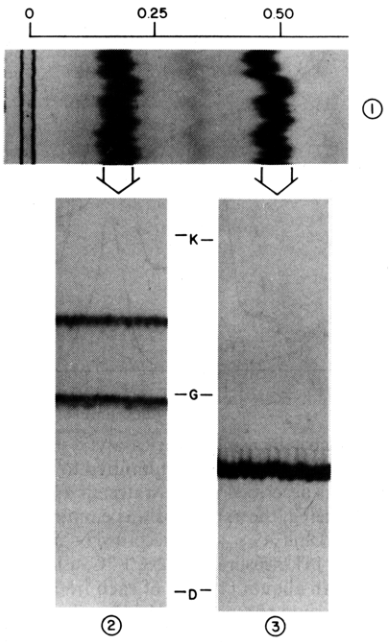

FIGURE 3: Fingerprint of the cysteine-containing peptides from the catalytic subunit of protein kinase. *S*-[^{14}C]Cyano catalytic subunit (50 nmol) was digested by pepsin (E/S = 0.1) in 1 mL of formic acid–acetic acid–water (1:4:45) during 18 h at 37 °C. A small insoluble core was discarded after centrifugation, and the clear supernatant was loaded directly on Whatman No. 3MM (20 nmol/cm) and submitted to a descending chromatography (18 h), in 1-butanol–pyridine–acetic acid–water (45:30:9:36). After drying, the paper was exposed to x-ray film for 48 h. (1) Numbers indicate R_f values. Radioactive bands of R_f ca. 0.2 and 0.5 were cut out and stitched across Whatman No. 3MM paper. High-voltage electrophoresis was carried out at 60 V/cm for 30 min, at pH 6.5 (pyridine–acetic acid–water, 25:1:125). 2 and 3 are the autoradiograms corresponding to the peptides of R_f ca. 0.2 and ca. 0.5, respectively. G, K, and D indicate the migration of glycine, lysine, and aspartic acid. When computed using aspartic acid as a standard, the mobility of the neutral and basic peptides was ca. 0 and −0.39, respectively (2). The mobility of the acidic peptide (3) was 0.38.

Composition and Properties. The catalytic subunit contains three half-cystine residues, all free since they can be entirely titrated in the denatured molecule by reaction with Nbs_2. Furthermore, three major radioactive peptides were detected in a peptide map of a peptic digest of the S-[^{14}C]cyano derivative of the C subunit (Figure 3). The spectrum (not illustrated)[2] shows a maximum at 280 nm ($A_{cm}^{1\%}$ = 14.9, ϵ = 57

[2] Submitted to the reviewers for examination. Will be provided to the interested reader by writing to the authors.

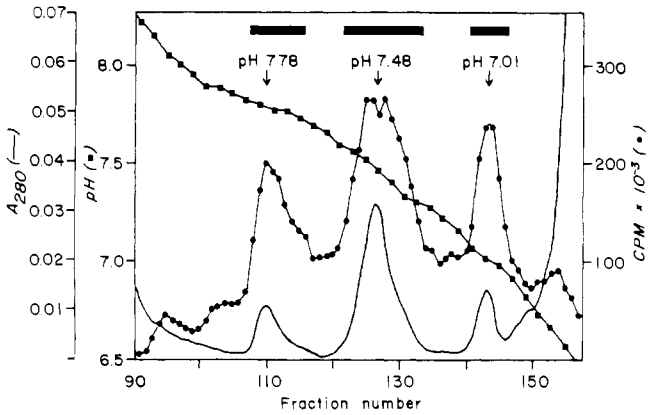

FIGURE 4: Isoelectric focusing of the catalytic subunit of protein kinase. Catalytic subunit (0.25 μmol) was submitted to isoelectric focusing in a sucrose gradient as described under Materials and Methods. After completion of the focusing, the apparatus was emptied through a cylindrical UV cell (3 mm i.d.) and $A_{280\ nm}$ was recorded (—). Fractions of 2 mL were collected and their pH measured at 22 ● 1 °C, using a phosphate standard of pH 7.01 (■). An aliquot (10 μL) of each fraction was mixed with 10 μL of 0.25 M potassium phosphate (pH 7.0) and assayed for protein kinase activity (●). An activity of 1 pmol min^{-1} would correspond, under these conditions, to 17 000 cpm. The fractions corresponding to the three main peaks were pooled as indicated (▬).

mM^{-1} cm^{-1}) and a marked minimum at 250 nm resulting in a high 280-250 nm absorbancy ratio of 2.96; these data are in perfect agreement with the value computed from the tryptophan (7 residues), phenylalanine, and tyrosine content.

The catalytic subunit contains no carbohydrate. Although no incorporation of acid- or alkali-stable phosphate could be demonstrated at neutral pH from [γ-^{32}P]MgATP, the enzyme contains 1.7 ± 0.2 ($\bar{X}$ + SD, n = 15) mol of acid-stable endogenous phosphate/mol of protein.

Amino Terminus. C subunit (130 nmol) was submitted to Edman degradation in the sequencer; no Pth amino acid could be identified in the first six cycles, using both gas- and high-pressure liquid chromatography, indicating that the NH_2 terminus is blocked. The procedure would have detected the presence of 1 nmol of free amino-terminal group.

Isozymes of the Catalytic Subunit. When submitted to isoelectric focusing (Figure 4), the catalytic subunit of bovine heart protein kinase displayed three major peaks corresponding to pI = 7.78, 7.48, and 7.01; other minor peaks or shoulders corresponding to pI = 8.05, 7.87, and 6.62 were also detected. Only the three major components were further characterized in terms of size and charge differences.

The migration of the three isozymes was exactly the same in 0.1% dodecyl sulfate–15% polyacrylamide gel, pointing to identical molecular weight. As indicated above, none of them exhibited a free NH_2 terminus, or the presence of carbohydrates. The attractive possibility that they might differ by the amount of bound phosphate was also ruled out (Table I). Their UV spectra showed no significant differnce. Attempts at demonstrating a difference in amino acid composition failed to provide an unequivocal answer. While histidine and lysine contents seem identical in the three species, one more arginyl residue appears to be present in the most basic (pI = 7.78) component whereas the most acidic form (pI = 7.01) seems to contain 1 additional Asx and 2 more Glx. It should be kept in mind, of course, that the free dicarboxylic acids and their amides are not discriminated here and that the composition differences fall close to the 3% error usually encountered in amino acid analysis.

Direct demonstration of the charge differences by polyacrylamide gel electrophoresis in the absence of dodecyl sulfate was not possible because of the poor migration of the C subunit even in the presence of 8 M urea; the gel pattern was further complicated by the appearance of multiple bands resulting from aggregation, as described above.

TABLE I: Charged Groups in the Major Isozymes of the Catalytic Subunit.

	Isoelectric point		
	7.01	7.48	7.78
Residues/mol[a]			
Asx	32.0 ± 0.2	30.8 ± 1.6	30.8 ± 0.3
Glx	37.4 ± 0.6	35.1 ± 1.2	34.7 ± 0.3
His	7.6 ± 0.1	7.2 ± 0.7	7.6 ± 0.1
Lys	28.6 ± 0.2	28.0 ± 1.7	28.7 ± 0.5
Arg	13.4 ± 0.2	12.8 ± 0.4	14.3 ± 0.2
Carbohydrate	<d.l.	<d.l.	<d.l.
Acid-stable phosphate	1.4 ± 0.3	1.5 ± 0.3	1.8 ± 0.1
α-NH_2 terminus	Blocked	Blocked	Blocked

[a] Figures from amino acid analysis are $\bar{X}$ ± SD (triplicates from 24-, 48-, and 72-h hydrolysis). Values were obtained assuming 24 phenylalanyl residues/mol. Carbohydrates were below detection limit (<d.l.). Standards for protein-bound phosphate were ovalbumin (1.4 ± 0.2 mol/mol, n = 12) and bovine serum albumin (no protein-bound phosphate).

Role of Sulfhydryl Groups in Catalysis. All three thiol groups of the C subunit could be titrated by Nbs_2 under nondenaturing conditions at 23 °C and at neutral pH. When a ca. ninefold molar excess of Nbs_2 over C subunit was added, the rates of reaction of the three sulfhydryl groups were different enough to be analyzed separately.

Graphical determination (not illustrated)[2] shows that a first group reacts very rapidly ($t_{1/2}$ = 0.36 min, k = 1.9 min^{-1}), and its substitution is often accompanied by a pronounced decrease in the solubility of the enzyme, especially when the solution is being manipulated by pipet transfers, etc. Variations in enzyme activity with time were therefore difficult to assess by this procedure. Using another approach to determine which SH group is essential for catalytic activity, limited amounts of Nbs_2 were added to aliquots of C subunit. Reaction was essentially complete after 3 h at 23 °C (Figure 5). From the nonsuperposition of the curves relative to Nbs_2 added on the one hand, and Nbs^- formed (i.e., SH titrated) on the other hand, it is obvious that disulfide formation occurs, with a final liberation of 2 mol of Nbs^- from 1 mol of Nbs_2. This can be expected when the concentration of Nbs_2 is lower than that of SH groups (Habeeb, 1972). In any event, it is clear that the most reactive sulfhydryl group can be titrated with less than a 10% loss in activity. Activity decreases as the second and third SH groups are titrated; approximately 40% of activity remains when 2 groups have been substituted and essentially total inhibition occurs after titration of 2.8 groups. Inactivation by Nbs_2 is completely reversed upon thiolysis (Table II).

The fact that substitution of both the second and third SH groups is required for the loss of activity argues against a specific involvement of these side chains in catalysis. This was confirmed by the following set of experiments.

First, the C subunit could be substituted with 3.0 equiv of [^{14}C]cyanide, while still retaining 63% of its original activity (see Table II). A peptide map of the S-[^{14}C]cyano C subunit further confirmed that all three cysteinyl residues had been modified (see Figure 3). It thus appears that the presence of a small substituent on the SH groups does not impair the catalytic properties of the molecule. Second, the protein kinase inhibitor, which acts competitively with respect to protein substrates (Demaille et al., 1977), is as effective on the native

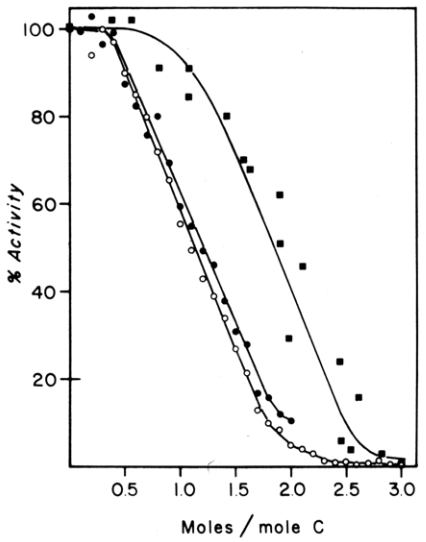

FIGURE 5: Inactivation of catalytic subunit by reaction with Nbs_2. (○ and ●) Activity plotted vs. mol of Nbs_2 added per mol of C subunit. C subunit (20 μM in 30 mM potassium phosphate, 1 mM EDTA, 0.15 M KCl, pH 7.0, 60 μL final volume) was incubated with increasing concentrations of Nbs_2 from 0 to 60 μM, by increments of 2 μM. After 3 h at 23 °C (●) and after 4 h at 23 °C plus 18 h at 4 °C (○), aliquots were diluted 150-fold and 20 μL of this dilution was then assayed. (■) Activity plotted vs. mol of SH titrated per mol of C subunit. The experiment was carried out as above in a final volume of 480 μL. After 4 h at 23 °C, absorbance was measured at 412 nm and enzyme activity was assayed. Scattering of light, variable from sample to sample after reaction with Nbs_2, is responsible for some scattering in points.

TABLE II: Substitution of the Thiol Groups of the Catalytic Subunit and Effect on Catalytic Activity.

Bound ligand	Stoichiometry (mol of ligand/mol of enzyme)	% residual act.
None		100
Nbs	3.0	1
Nbs after thiolysis[a]		100
[^{14}C]CN	3.0	63
[^{14}C]CN after thiolysis[b]		
Minus Gdn·HCl	0.7	100
Plus Gdn·HCl	0.07	

[a] Catalytic subunit (18.5 μM) was reacted with Nbs_2 and the extent of substitution determined by absorbance at 412 nm; it was assayed before and after incubation at 30 °C, 30 min, with 1 mM dithiothreitol. [b] Thiolysis was performed on 1 mL of *S*-[^{14}C]cyano catalytic subunit solution (108.7 μg of protein, containing 3.0 mol of [^{14}C]-CN/mol of C subunit) incubated with 150 mM 2-mercaptoethanol for 75 min at 30 °C. Thiolysis under denaturing conditions was performed in the presence of 6 M Gdn·HCl.

as on the fully cyanylated enzyme, showing that the substrate binding site remains essentially intact (Figure 6A). As described for the Nbs_2 derivative, the S-cyanylated enzyme recovers full activity upon thiolysis under nondenaturing conditions (Table II) even though 0.7 mol of cyanide/mol of protein still remains attached. This last cyano group is only displaced upon thiolysis in 6 M Gdn·HCl, confirming that 1 out of the 3 cysteinyl side chains (probably the slow reacting one) is not readily accessible to solvent in the native molecule.

Third, the *S*-cyano C subunit containing either 3 or 0.7 equiv of cyanide was further reacted with iodoacetamide (Figure 6B). Whereas this treatment brought about a 90% loss of activity in both the native enzyme and the derivative that contained only 0.7 mol of CN/mol of protein, the percyanylated protein was almost entirely protected against iodoacetamide inactivation.

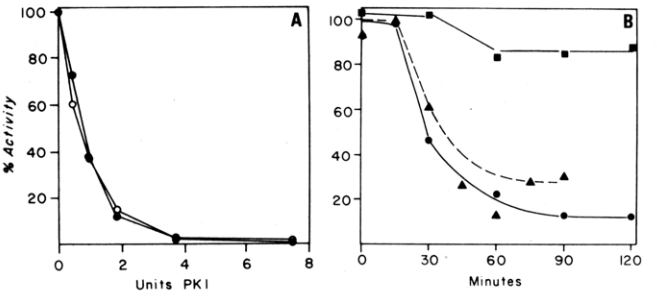

FIGURE 6: (A) Inhibition of the catalytic subunit and of its *S*-cyano derivative by the protein kinase inhibitor. (●) Native catalytic subunit (20 μL, 5.4 μg/mL) or (○) *S*-cyano catalytic subunit (20 μL, 5.3 μg/mL containing 3 mol of CN/mol) in 30 mM potassium phosphate, 1 mM EDTA, 0.15 M KCl, pH 7.0, are assayed with increasing amounts of PKI in a total volume of 90 μL. A unit of PKI is defined in Demaille et al., (1977). (B) Effect of iodoacetamide on the activity of catalytic subunit in various states of cyanylation. Native (●) or percyanylated (3 mol of CN/mol) catalytic subunit (■) (9.6 μM in 30 mM potassium phosphate, 1 mM EDTA, 0.15 M KCl, pH 7.0) was incubated at 23 °C in the dark with and without 200 μM iodacetamide. Mono-*S*-cyano catalytic subunit (0.7 mol of CN/mol) (▲) (105 nM in the above buffer) was assayed by addition of 50 μL of the histone–MgATP mixture containing 0.15 M 2-mercaptoethanol.

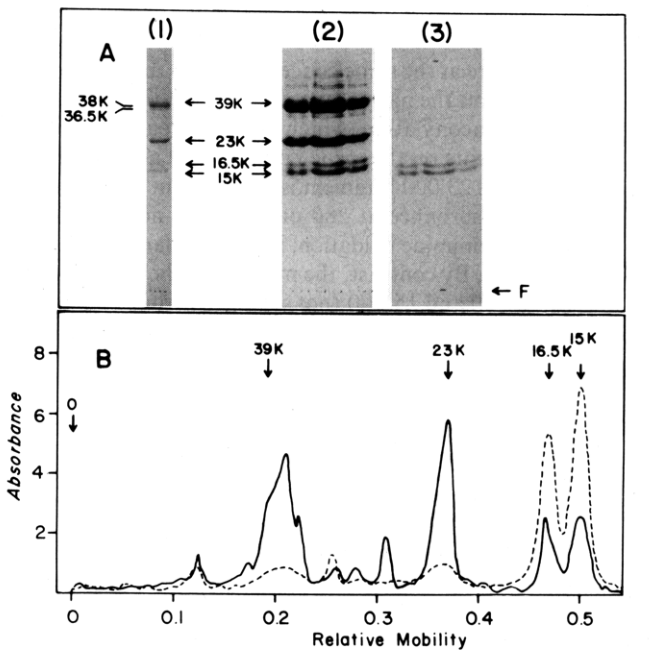

FIGURE 7: Analysis of cleavage products of *S*-[^{14}C]cyano catalytic subunit by 0.1% dodecyl sulfate–15% polyacrylamide gel electrophoresis, fluorography, and densitometry. (A) Gel electrophoresis of (1) 10.6 μg of the cleaved *S*-[^{14}C]cyano C subunit showing clearly the three upper minor bands (see text) and (2) 53, 80, and 21 μg, respectively, of the cleaved protein; (3) is the fluorogram of gel (2). (B) Densitometric traces of (—) the Coomassie stained gel depicted in A (2), and (- - -) its fluorogram, A (3): O, origin; F, dye front.

Cleavage of the S-Cyano C Subunit. *S*-[^{14}C]Cyano C subunit (400 μg) was cleaved in 1% dodecyl sulfate, 0.2 M sodium borate, pH 9.0, and the cleavage products were separated by 0.1% dodecyl sulfate–15% polyacrylamide gel electrophoresis (Figure 7). Very little of the original 39 000 component remained; two new bands of mol wt 38 000 and 36 500 were visible although not clearly resolved in the densitometric tracing. The major fragments corresponded to

molecular weights of 23 000, 16 500, and 15 000. Assuming equivalent Coomassie binding for each fragment, densitometry of the stained gel and of its fluorogram showed that the 23 000 component was generated in a 60% yield. Only the 16 500 and 15 000 peptides were radioactive, i.e., blocked by a 2-iminothiazolidine-4-carboxylyl derivative at their NH_2 terminus (35 and 46% of total protein radioactivity, respectively). The other fragments (38 000, 36 500, and 23 000 mol wt) exhibited negligible radioactivity (e.g., only 9% of total radioactivity for the 23 000 component) suggesting that they originated from the NH_2 terminus of the original molecule. In support of this hypothesis, it was found that for every mol of 23 000 peptide, 1 mol of (16 500 + 15 000) component was generated. The amino acid compositions (Table III) of the 16 500 and 15 000 fragments eluted from preparative acrylamide gel electrophoresis were very similar, leaving little doubt that they originated from the same portion of the peptide chain.

In order to assess the potential usefulness of the cleavage of the *S*-cyano catalytic subunit, the fragments were separated on Sephadex G-150 in 0.1% dodecyl sulfate, 50 mM sodium borate at pH 9.0. In spite of these denaturing conditions, the cleaved enzyme emerged in a single peak, as checked by dodecyl sulfate-polyacrylamide gel electrophoresis. The molecule was thus only "nicked" and fragments still held together under these conditions. It was, however, totally inactive, whereas dodecyl sulfate at the same concentration in the assay mixture does not inhibit the native enzyme. Dissociation was brought about by citraconylation, which allowed a partial separation of the fragments on the same column. The amino acid composition of the 23 000 fragment is presented in Table III. It has a low UV absorbance at 280 nm which is not decreased by *N*-bromosuccinimide oxidation; its tryptophan content is thus probably low. By contrast, the mixture of the polypeptides of mol wt 16 500 and 15 000 (not separated from each other on the Sephadex column) strongly absorbed in the ultraviolet.

TABLE III: Amino Acid Composition of the Catalytic Subunit and of Its Fragments.

	Catalytic subunit[a] (residues/mol)	Mol wt 23 000[b] (residues/mol)	Residues per 100 residues	
			Mol wt 16 500[c]	Mol wt 15 000[c]
	Integer			
Asx	32	19.6	13.7	14.9
Thr[d]	14	9.2	4.3	3.8
Ser[d]	16	11.0	7.0	6.3
Glx	39	29.9	16.2	16.0
Pro	14	10.1	8.7	9.0
Gly	21	13.8	7.4	7.0
Ala	22	12.8	5.2	5.5
Val[e]	19	11.2	4.9	5.1
Met[f]	7	2.8	0.5	0.4
Ile[e]	19	8.7	6.5	6.9
Leu	29-30	17.6	6.3	6.3
Tyr	12-13	4.5	1.9	1.9
Phe	24	10.0	5.5	5.1
His	9	4.2	1.1	1.0
Lys	31	19.5	9.3	10.0
Trp[g]	7	n.d.		
Arg	14-15	4.8	1.4	0.9
Cys[h]	3			
	332-335			

[a] Taken from Demaille et al. (1977). [b] The 23 000 component was isolated on Sephadex G-150 from a citraconylated cleavage mixture (see Materials and Methods), then dialyzed vs. 0.2 M acetic acid, lyophilized, and hydrolyzed for 24, 48, and 72 h. [c] The peptides of mol wt 16 500 and 15 000 were eluted from a preparative dodecyl sulfate-polyacrylamide gel (see Materials and Methods) and hydrolyzed for only 24 h. Values are therefore expressed only as residues/100 residues and given for the sole purpose of comparison of the 2 peptides. Methionine was probably destroyed by persulfate. [d] After extrapolation to zero time of hydrolysis, or, for the peptides of mol wt 16 500 and 15 000, after correction for 5% (threonine) and 10% (serine) destruction. [e] From the 72-h hydrolysis value, except for peptides of mol wt 16 500 and 15 000. [f] For fragment of mol wt 23 000, computed from the sum of methionine + homoserine + homoserine lactone. [g] As determined after alkaline hydrolysis of the catalytic subunit. [h] As cysteic acid. The 23 000 fragment is NH_2 terminal and thus contains no Cys. The smaller fragments are 2-iminothiazolidine-4-carboxylyl peptides.

Discussion

It is possible to prepare, within 1 week, micromolar amounts of the catalytic subunit from bovine heart by the protocol described earlier (Beavo et al., 1974; Bechtel et al., 1977; Demaille et al., 1977) as modified herein. The isolated catalytic subunit is less stable than the holoenzyme and, in particular, exhibits a strong tendency to aggregate at low ionic strength and neutrality (i.e., close to the isoelectric point). Such a polymerization has been reported by Sugden et al. (1976) for the bovine liver enzyme.

The properties of the catalytic subunit from the bovine heart enzyme are very similar to those of the protein recently isolated from bovine liver (Sugden et al., 1976) and rabbit skeletal muscle (Bechtel et al., 1977) including the length and composition of their polypeptide chains, and their UV adsorbance. The major difference lies in the cysteine content: three half-cystines are present in the skeletal and cardiac muscle enzymes, whereas only one has been reported for the bovine liver protein (Sugden et al., 1976). A detailed study of acid-stable, protein-bound phosphate has been made on the rabbit skeletal muscle C subunit (Bechtel et al., 1977); slightly lower amounts were found in the heart enzyme. No incorporation of ^{32}P from [γ-^{32}P]ATP was observed even when precautions were taken to avoid hydrolysis of acid-labile phosphohistidine bonds (Kochetkov et al., 1976). It is, of course, possible that another kinase is responsible for the phosphorylation of the C subunit (as suggested by Bechtel et al., 1977).

As structural studies are now rendered possible by the availability of mass purification procedures, it was of particular importance to ascertain the nature and origin of the isozymes found in C subunit preparations (Sugden et al., 1976; Bechtel et al., 1977). The bovine heart enzyme was also shown to contain at least three main isozymes. As of now, analytical studies have failed to reveal marked differences in composition (except, perhaps, for some differences in the arginine, Asx, and Glx content). Since their NH_2 termini are blocked, one will have to rely on sequence studies, now under way, to find the origin of such differences in isoelectric point. In any case, the isozymes have been reported to exhibit identical substrate specificity (Bechtel et al., 1977). The three thiol groups appear to be located in homologous positions within the peptide chain in view of the simplicity of the cleavage pattern. It can thus be assumed that the isozymes will differ only slightly from one another.

Bovine heart C subunit can be inactivated by modification of its thiol groups with bulky (nitrothiobenzoate, carboxamidomethyl) substituents. However, in contrast to the bovine liver and skeletal muscle enzymes (Sugden et al., 1976; Bechtel et al., 1977) which are inactivated after binding 1 equiv of reagent, the bovine heart C subunit exhibits one very reactive thiol group, which can be substituted without significant loss of activity. Presumably, it is distant from the active site. Inhibition occurs when the second group, probably located in the

vicinity of the active site, and the third group, almost buried, are substituted. None of the thiols is involved in catalysis since the enzyme retains 63% of its original activity when percyanylated. This situation is reminiscent of that described for the catalytic subunit of aspartate transcarbamoylase (Vanaman and Stark, 1970) and for creatine kinase as seen either after methylation (Maggio et al., 1977; Markham et al., 1977) or cyanylation (Der Terrossian and Kassab, 1976) of the sulfhydryl groups. In all instances, introduction of a small substituent leaves intact a substantial portion of the enzyme activity.

Substitution of the thiol group seems to alter the nucleotide binding site of creatine kinase (Maggio et al., 1977). A similar situation might prevail with the bovine liver C subunit which is protected from iodoacetamide inactivation by MgATP (Sugden et al., 1976). Likewise, the protein–substrate binding site of the percyanylated bovine heart enzyme seems to be essentially intact as probed by the use of the protein kinase inhibitor. It is thus possible that the second SH group ($k = 0.2$ min^{-1}) is close to, if not part of the nucleotide binding site.

Cyanylation of the molecule also offers the possibility of cleaving the molecule into large fragments since cysteine is the least abundant residue, and no other bond (Asp-Pro, Asn-Gly) used for this purpose was found to be present. Among the cleavage products, the 23 000 component is presumably NH_2 terminal; as shown by its methionyl and arginyl contents, it should provide, after CNBr or tryptic cleavage, large peptides suitable for sequence studies. The 16 500 and 15 000 polypeptides are probably derived from the COOH-terminal half of the molecule. Finally, since the most reactive thiol group can be blocked even by a bulky substituent without loss of activity, thiol-Sepharose could probably be conveniently used to immobilize the C subunit (Carlsson et al., 1975) in contrast to CNBr-activated Sepharose which results in 80% loss of activity (Demaille et al., 1977).

Acknowledgments

The authors are grateful to T. P. Strandjord and to C. D. Diltz for help in preparing catalytic subunit, to L. H. Ericsson for analyzing the NH_2 terminus of the enzyme, to R. Granberg for carrying out the amino acid analyses, and to Cheryl May for typing this manuscript.

References

Ames, B. N. (1966), *Methods Enzymol. 8,* 116.
Atassi, M. Z., and Habeeb, A. F. S. A. (1972), *Methods Enzymol. 25,* 546.
Beavo, J. A., Bechtel, P. J., and Krebs, E. G. (1974), *Methods Enzymol. 38,* 299.
Bechtel, P. J., Beavo, J. A., and Krebs, E. G. (1977), *J. Biol. Chem. 252,* 2691.
Bonner, W. M., and Laskey, R. A. (1974), *Eur. J. Biochem. 46,* 83.
Carlsson, J., Axen, R., and Unge, T. (1975) *Eur. J. Biochem. 59,* 567.
Degani, Y., and Patchornik, A. (1974), *Biochemistry 13,* 1.
Demaille, J. G., Peters, K. A., and Fischer, E. H. (1977), *Biochemistry 16,* 3080.
Der Terrossian, E., and Kassab, R. (1976), *Eur. J. Biochem. 70,* 623.
Ellman, G. L. (1959), *Arch. Biochem. Biophys. 82,* 70.
Erlichman, J., Rubin, C. S., and Rosen, O. M. (1973), *J. Biol. Chem. 248,* 7607.
Gill, G. N., Holdy, K. E., Walton, G. M., and Kanstein, C. B. (1976), *Proc. Natl. Acad. Sci. U.S.A. 73,* 3918.
Glossmann, H., and Neville, D. M., Jr. (1971), *J. Biol. Chem. 246,* 6339.
Glynn, I. M., and Chappell, J. B. (1964), *Biochem. J. 90,* 147.
Habeeb, A. F. S. A. (1972), *Methods Enzymol. 25,* 457.
Hermodson, M. A., Ericsson, L. H., Titani, K., Neurath, H., and Walsh, K. A. (1972), *Biochemistry 11,* 4493.
Hirs, C. H. W. (1967), *Methods Enzymol. 11,* 59.
Hofmann, F., Beavo, J. A., Bechtel, P. J., and Krebs, E/ G. (1976), *J. Biol. Chem. 250,* 7795.
Hofmann, F., Bechtel, P. J., and Krebs, E. G. (1977), *J. Biol. Chem. 252,* 1441.
Hugli, T. E., and Moore, S. (1972), *J. Biol. Chem. 247,* 2828.
Itaya, K., and Michio, U. (1966), *Clin. Chim. Acta 14,* 361.
Jacobson, G. R., Schaffer, M. H., Stark, G. R., and Vanaman, T. C. (1973), *J. Biol. Chem. 248,* 6583.
Kochetkov, S. N., Bulargina, T. V., Sashchemko, L. P., and Severin, E. S. (1976), *FEBS Lett. 71,* 212.
Laemmli, U. K. (1970), *Nature (London) 227,* 680.
Lincoln, T. M., Dills, W. L., and Corbin, J. D. (1977), *J. Biol. Chem. 252,* 4369.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193,* 265.
Maggio, E. T., Kenyon, G. L., Markham, G. D., and Reed, G. H. (1977), *J. Biol. Chem. 252,* 1202.
Markham, G. D., Reed, G. H., Maggio, E. T., and Kenyon, G. (1977), *J. Biol. Chem. 252,* 1197.
Moore, S., and Stein, W. H. (1963), *Methods Enzymol. 6,* 819.
Pechère, J. F., Damaille, J., and Capony, J. P. (1971), *Biochim. Biophys. Acta 236,* 391.
Rangel-Aldao, R., and Rosen, O. M. (1976a), *J. Biol. Chem. 251,* 3375.
Rangel-Aldao, R., and Rosen, O. M. (1976b), *J. Biol. Chem. 251,* 7526.
Reisfeld, R. A., Lewis, U. J., and Williams, D. E. (1962), *Nature (London) 195,* 281.
Rosen, O. M., and Erlichman, J. (1975), *J. Biol. Chem. 250,* 7788.
Rubin, C. S., and Rosen, O. M. (1975), *Annu. Rev. Biochem. 44,* 831.
Sugden, P. H., Holladay, L. A., Reimann, E. M., and Corbin, J. D. (1976), *Biochem. J. 159,* 409.
Vanaman, T. C., and Stark, G. R. (1970), *J. Biol. Chem. 245,* 3565.
Walsh, D. A., and Krebs, E. G. (1973), *Enzymes, 3rd Ed. 8,* 555.