Modification and Identification of Glutamate Residues at the Arginine-Recognition Site in the Catalytic Subunit of Adenosine 3':5'-Cyclic Monophosphate-Dependent Protein Kinase of Rabbit Skeletal Muscle

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It has been proposed that the active centre of cyclic AMP-dependent protein kinase contains an arginine-recognition site, which is considered to be essential for the function of the catalytic subunit of the kinase [Matsuo, Huang & Huang (1978) *Biochem. J.* **173**, 441–447]. The catalytic subunit can be inactivated by 3-(3-dimethylaminopropyl)-1-ethylcarbodi-imide and glycine ethyl ester at pH 6.5. The enzyme can be protected from inactivation by preincubation with histone, a protein substrate of the enzyme. On the other hand, ATP, which also serves as a protein kinase substrate, does not afford protection. Polyarginine, a competitive inhibitor of protein kinase, which is known from kinetic studies to interact specifically with the arginine-recognition site, partially protects the catalytic subunit from inactivation by 3-(3-dimethylaminopropyl)-1-ethylcarbodi-imide. These results lead to the conclusion that the site of modification by carbodi-imide/glycine ethyl ester is most likely located at the arginine-recognition site of the active centre. A value of 1.7 ± 0.2 (mean $\pm$ s.d.) mol of carboxy groups per mol of catalytic subunit has been obtained for the number of essential carboxy groups for the function of protein kinase; a complete chemical modification of these essential carboxy groups results in total loss of catalytic activity. Finally, we have identified the essential carboxy group in the catalytic subunit of cyclic AMP-dependent protein kinase as being derived from glutamate residues. This is achieved by a three-step procedure involving an extensive proteolytic digestion of the [$1\text{-}^{14}\text{C}$]glycine ethyl ester-modified enzyme and two successive high-voltage electrophoreses of the hydrolysate. It is concluded that 1.7 mol of glutamyl carboxy groups per mol of catalytic subunit may be considered a component of the arginine-recognition site in the active centre of cyclic AMP-dependent protein kinase.

It has become increasingly evident that two adjacent basic amino acid residues on the *N*-terminal side of the phosphorylatable serine or threonine residues in the amino acid sequence of protein substrates are crucial for the function of cyclic AMP-dependent protein kinase (Zetterqvist *et al.*, 1976; Cohen *et al.*, 1977; Proud *et al.*, 1977; Shenolikar & Cohen, 1978). The importance of these basic amino acid residues is further substantiated by studies with synthetic substrates (Kemp *et al.*, 1977). It is noteworthy that one of these two basic amino acid residues in protein substrates is gener-

ally arginine at the third residue from the site phosphorylated by cyclic AMP-dependent protein kinase (Matsuo *et al.*, 1978*b*); this vicinal arginine residue has since been shown kinetically to be critical for the enzyme–substrate interaction (Matsuo *et al.*, 1978*b*; Kemp, 1979). Accordingly, an arginine-recognition site near the phosphorylation site in the kinase active centre has been suggested. This arginine-recognition site on cyclic AMP-dependent protein kinase is considered to be a distinct functional part of the active centre, because not only does it act as a recognition site that participates in binding the specific arginine residue on the protein substrate, but also it may help to steer the adjacent serine or threonine residue on the substrate surface

Abbreviations used: Mes, 4-morpholine-ethanesulphonic acid; Pipes, 1,4-piperazinediethanesulphonic acid.

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into a proper orientation on the nearby phosphorylation site for the phosphotransferase reaction (Matsuo *et al.*, 1978b).

In an attempt to identify the arginine-recognition site on the catalytic subunit of cyclic AMP-dependent protein kinase (mol.wt. 41 000), we have carried out the chemical modification of carboxylic acid groups of the catalytic subunit of the enzyme. The design of these experiments was based on the structural properties of arginine. First, the positively charged guanidinium ion of arginine is a relatively flat large moiety capable of extending over a considerable distance. Secondly, the cationic guanidinium group is also an obligatory hydrogen donor, and it can form as many as five hydrogen bonds (Cotton *et al.*, 1971). Consequently, a particularly good co-operative bond might be made between the cationic guanidinium group of the protein substrate and a negatively charged carboxy group of the enzyme, through complementary electrostatic interaction and hydrogen bonds. It is therefore tempting to assume that carboxy groups may be an essential component of the arginine-recognition site in the catalytic subunit of cyclic AMP-dependent protein kinase. Our speculation that the arginine-recognition site may contain carboxy groups was tested experimentally as described in the present paper. Kinase activities were correlated with the specific modification of carboxy groups in the protein kinase as a function of time. Moreover, γ -carboxy groups of glutamate residues were identified as those implicated in the arginine-recognition site.

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Experimental

Materials

Histone type IIA and 3-(3-dimethylaminopropyl)-1-ethylcarbodi-imide were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Trypsin, carboxypeptidase A and carboxypeptidase B were purchased from Worthington Biochemical Corp., Freehold, NJ, U.S.A. Pronase was from Calbiochem, La Jolla, CA, U.S.A. Leucine aminopeptidase was from Boehringer–Mannheim, New York, NY, U.S.A. γ -L-Glutamylglycine (lot no. 051246) was purchased from Vega–Fox Biochemicals, Tucson, AZ, U.S.A. β -L-Aspartylglycine was a gift from Dr. R. F. Colman, University of Delaware. Radioactive orthophosphoric acid ($[^{32}\text{P}]\text{P}_i$) was obtained from New England Nuclear, Boston, MA, U.S.A., which was used to prepare $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ by the method of Glynn & Chappell (1964). $[1\text{-}^{14}\text{C}]\text{Glycine}$ ethyl ester hydrochloride (sp. radioactivity 41.59 mCi/mmol, lot no. 937–218) was obtained from New England Nuclear. Instant t.l.c. sheets of

glass microfibre were purchased from Fisher, Pittsburgh, PA, U.S.A. All other chemicals were of analytical or reagent grades. Water was deionized and glass-distilled.

Enzyme assay and purification

Cyclic AMP-dependent protein kinase activity was assayed radioisotopically as described elsewhere (Huang, 1974; Huang & Huang, 1975). The catalytic subunit of cyclic AMP-dependent protein kinase (type I) was purified to homogeneity from rabbit skeletal muscle, by using a modified procedure (Matsuo *et al.*, 1978b) of that of Bechtel *et al.* (1977). Before chemical modifications, the purified subunit was dialysed exhaustively against 5 mM-Mes buffer, pH 6.5, to remove 2-mercaptoethanol.

Reaction of the carbodi-imide derivative with the catalytic subunit of cyclic AMP-dependent protein kinase

Enzyme was incubated at room temperature in 50 mM-Mes buffer (pH 6.5) with 200 mM-glycine ethyl ester in the absence and presence of various concentrations of 3-(3-dimethylaminopropyl)-1-ethylcarbodi-imide as specified in the relevant Figure legend; the final volume of this incubation mixture was 0.1 ml. At desired times, a portion (2 μl) of the mixture was transferred to the protein kinase assay mixture (90 μl) to determine the residual protein kinase activity. To minimize the effect of 3-(3-dimethylaminopropyl)-1-ethylcarbodi-imide on kinase during the time course of the assay, the kinase-catalysed reaction was allowed to proceed for only 2.5 min. In general, the activity was expressed as the percentage of the initial activity in the presence and in the absence of carbodi-imides as given in the legend to Fig. 1(a).

$[1\text{-}^{14}\text{C}]\text{Glycine}$ ethyl ester incorporation into the catalytic subunit

Enzyme was incubated in a reaction mixture containing 50 mM-Mes buffer (pH 6.5), 100 mM-3-(3-dimethylaminopropyl)-1-ethylcarbodi-imide and 80 μM - $[1\text{-}^{14}\text{C}]\text{glycine}$ ethyl ester (sp. radioactivity 41.59 mCi/mmol) for an indicated time. At the end of incubation, a portion of the mixture was spotted on a strip of instant t.l.c. paper (1.5 cm $\times$ 10 cm) previously treated with 20% trichloroacetic acid. After drying, the instant t.l.c. paper was developed with 5% trichloroacetic acid containing 0.2 M-KCl. Under these conditions, the denatured protein remained at the origin, whereas the unreacted glycine ethyl esters migrated with the solvent front. The origin was cut into a segment (1.5 cm $\times$ 2 cm) and the amount of radioactively labelled protein was determined by counting this segment for radio-

activity in Bray's solution with a Beckman LS 7000 liquid-scintillation counter (Bray, 1960).

Determination of the site of incorporation of [1-¹⁴C]glycine ethyl ester

Enzyme was incubated in 50mM-Mes buffer (pH 6.5) with 32mM-3-(3-dimethylaminopropyl)-1-ethylcarbodi-imide in the presence of 180mM-glycine ethyl ester containing 50 μ Ci of [1-¹⁴C]glycine ethyl ester for 20 min. After dialysis at 4°C against 5mM-Mes buffer (pH 6.5) to remove excess reagents, the samples were further dialysed exhaustively against 50mM-NH₄HCO₃ buffer (pH 7.8). They were subsequently digested at 40°C over a period of 4 days with sequential additions of the following proteolytic enzymes: trypsin (0.05 mg), which was added twice; Pronase (0.05 mg); carboxypeptidase A and B (0.01 mg each); leucine aminopeptide (0.01 mg), by the procedure of Ramachandran & Colman (1977). After proteolysis, the protein hydrolysate was divided into two samples (representing one-fifth and four-fifths of the total material respectively), and the samples were then subjected to high-voltage paper electrophoresis under a potential gradient of 34.5 V/cm in pyridine/acetate buffer (pH 6.4) for 55 min. The standard dipeptides β -aspartylglycine and γ -glutamylglycine were used as markers, and they were run parallel to the protein hydrolysate. The strip of the electrophoresis paper containing the smaller sample and standard markers were stained with ninhydrin reagent; the radioactivity of the paper strip was determined in Bray's solution in a Beckman LS 7000 liquid-scintillation counter. The corresponding major radioactive spot of the larger sample was eluted with water. The eluate was further subjected to high-voltage paper electrophoresis together with standard markers in pyridine/acetate buffer, pH 3.5, for 5 h at 2.5 kV. The paper was then stained with ninhydrin and the electrophoretogram was analysed for radioactivity.

Determination of thiol groups in protein kinase

The number of total thiol groups in the catalytic subunit of cyclic AMP-dependent protein kinase with and without 3-(3-dimethylaminopropyl)-1-ethylcarbodi-imide inactivation was measured in the presence of 6M-urea by the method of Ellman (1959). Two portions (0.8 ml) of the catalytic subunit (0.27 mg/ml) were preincubated in 50mM-Mes buffer (pH 6.5) at 25°C for 5 min. Then 0.2 ml of 1M-glycine ethyl ester and 0.03 ml of 1M-3-(3-dimethylaminopropyl)-1-ethylcarbodi-imide, pH 6.5, were added to one, whereas the other received 0.2 ml of 1M-glycine ethyl ester, pH 6.5, and 0.03 ml of water as control. Portions of the enzyme mixture (0.5 ml) were withdrawn after 10 and 20 min of incubation at 25°C. After an initial dialysis against

10mM-Mes containing 5% glycerol at pH 6.5, the enzyme was further dialysed against 0.1M-Tris/HCl buffer at pH 7.1. The dialysed enzyme (0.35 ml) was mixed with 0.5 ml of 10M-urea in 0.1M-Tris/HCl, pH 7.1, in a 1 ml cuvette. Before addition of the Ellman's reagent, the protein concentration of the enzyme mixture in the cuvette was measured by the spectrophotometric method of Kalb & Bernlohr (1977). Then 50 μ l of 50mM-EDTA in 6M-urea and 100 μ l of a fresh solution of 5,5'-dithiobis-(2-nitrobenzoate) (10mM) in 6M-urea was added to the cuvette. The increase in A_{412} of the enzyme solution with and without the 3-(3-dimethylaminopropyl)-1-ethylcarbodi-imide inactivation was recorded. The increase in absorbance over the reagent blank was used to calculate the number of thiol groups, using 13 600 litre $\cdot$ mol⁻¹ $\cdot$ cm⁻¹ as extinction coefficient.

Protein determination

Protein concentration was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as a standard.

Results

Reaction of the carbodi-imide derivative with the catalytic subunit of cyclic AMP-dependent protein kinase

Fig. 1(a) shows the loss of enzyme activity on incubation of the catalytic subunit of cyclic AMP-dependent protein kinase with 3-(3-dimethylaminopropyl)-1-ethylcarbodi-imide and glycine ethyl ester at pH 6.5. For each initial carbodi-imide concentration the inhibition of the enzymic activity was correlated exponentially with the incubation time allowed for the chemical modification. Moreover, the rate of loss of kinase activity was found to increase progressively with increasing concentration of the modifying reagent (Fig. 1a).

In addition to modification of carboxy groups in proteins, 3-(3-dimethylaminopropyl)-1-ethylcarbodi-imide can have side reactions at acidic pH with phenolic and thiol groups in proteins (Carraway & Koshland, 1972). However, if the modification reaction is carried out at alkaline pH, 3-(3-dimethylaminopropyl)-1-ethylcarbodi-imide can be considered as a specific reagent for modification of the phenolic hydroxy group of tyrosine residues in proteins (Grouselle & Pudles, 1977). Fig. 1(b) shows that at pH 8.0 the chemical modification of tyrosine residues has no effect on the kinase activity. These results indicate that the loss of kinase activity cannot be attributed to the side reaction of 3-(3-dimethylaminopropyl)-1-ethylcarbodi-imide with phenolic groups of the kinase catalytic subunit.

To rule out the possibility that the 3-(3-dimethylaminopropyl)-1-ethylcarbodi-imide inactivation is due to modification of the thiol group on

Fig. 1. Inactivation by carbodi-imide

(a) Shows inactivation of the catalytic subunit in the time course of modifications with various concentrations of 3-(3-dimethylaminopropyl)-1-ethylcarbodi-imide at pH 6.5. Purified catalytic subunit (8.4 μ g) was used in each of the experiments under the conditions described in the Experimental section. Results are expressed as the enzyme activities obtained after modification with 3-(3-dimethylaminopropyl)-1-ethylcarbodi-imide and glycine ethyl ester at time-interval t as a percentage of the initial activity. The perfect maximum activity (100%) at time t is based on the activity present in the sample that was not treated with 3-(3-dimethylaminopropyl)-1-ethylcarbodi-imide. Symbols representing the concentration of 3-(3-dimethylaminopropyl)-1-ethylcarbodi-imide are: ○, 0 mM; ●, 50 mM; □, 100 mM; ■, 150 mM. (b) Shows the effect of the pH on the inactivation by carbodi-imide. Purified catalytic subunit (8.4 μ g) was incubated with 100 mM-3-(3-dimethylaminopropyl)-1-ethylcarbodi-imide under standard conditions described in the Experimental section, except that a different buffer was used as indicated. Symbols: ▲, 50 mM-Tricine/HCl buffer at pH 8.0; △, 50 mM-Pipes buffer at pH 7.0; ●, 50 mM-Mes buffer at pH 6.5; ○, 50 mM-Mes buffer at pH 6.0.

were compared. The results show that under the experimental conditions the total number of thiol groups is 2.4 ± 0.1 (mean $\pm$ s.d.) mol per 41000-mol.wt. protein with or without 3-(3-dimethylaminopropyl)-1-ethylcarbodi-imide inactivation. The catalytic subunit of cyclic AMP-dependent protein kinase from rabbit skeletal muscle has been shown to contain three cysteine residues by Bechtel *et al.* (1977). Our value, 2.4 ± 0.1 , as determined with the Ellman reagent, might be due to partial oxidation of thiol groups of the enzyme during the process of removing the unreacted modifying reagents by the prolonged dialysis (see the Experimental section). Since there is no change in the thiol group content during the time course of the 3-(3-dimethylaminopropyl)-1-ethylcarbodi-imide inactivation reaction, it can be concluded that the inactivation of the protein kinase observed in Fig. 1 can be ascribed most likely to the interaction of 3-(3-dimethylaminopropyl)-1-ethylcarbodi-imide with the carboxy groups of the enzyme via the carbodi-imide-nucleophile reaction.

Effects of substrates on the inactivation of kinase by 3-(3-dimethylaminopropyl)-1-ethylcarbodi-imide

By preincubation of the catalytic subunit of cyclic AMP-dependent protein kinase with histone, a protein kinase substrate, at a concentration of 3.5 μ g/ μ g of the catalytic subunit, the subunit retained full activity during the time course of the chemical modification by 3-(3-dimethylaminopropyl)-1-ethylcarbodi-imide (Fig. 2). However, if the same concentration of histone was first mixed with the 3-(3-dimethylaminopropyl)-1-ethylcarbodi-imide reaction mixture and the enzyme was then added, the loss of kinase activity was observed. These experiments strongly suggest that histone, if it is preincubated with the enzyme, protects those carboxy groups of the enzyme involved in phosphorylation from the modification by 3-(3-dimethylaminopropyl)-1-ethylcarbodi-imide. In contrast, the kinase activity was not protected by ATP even when the molar concentration of ATP was about 10 times greater than that of kinase (Fig. 2). This result suggests that the ATP-binding site in the enzyme active centre does not contain residues having carboxy groups. It should be pointed out here that in these experiments 5 μ l of histone or ATP was preincubated for 10 min with 75 μ l of kinase at a concentration of 3.5 μ g of histone per μ g of the catalytic subunit before the 3-(3-dimethylaminopropyl)-1-ethylcarbodi-imide reaction mixture was added. In the case of ATP, the concentration used was 7.5 mol/mol of enzyme. In the parallel control experiment, the substrate (histone or ATP) was not used. Instead, the enzyme was preincubated with Mes buffer. Because of the omission of glycine ethyl ester, relative kinase activities obtained in the control experiment shown in the bottom curve of Fig. 2

the enzyme, the total numbers of thiol groups on the protein kinase with and without 3-(3-dimethylaminopropyl)-1-ethylcarbodi-imide modification

Fig. 2. Effects of substrate on the inactivation by carbodi-imide

Catalytic subunit (8.4 μ g) was preincubated with histone IIA or ATP at 0°C for 10 min in a total volume of 80 μ l; then the mixture was transferred to a tube containing 20 μ l of 250 mM-Mes (pH 6.5) containing 0 or 500 mM-3-(3-dimethylaminopropyl)-1-ethylcarbodi-imide, but no glycine ethyl ester. This mixture was then incubated at room temperature. At indicated times, a portion (2 μ l) was removed and its protein kinase activity was determined as described in the Experimental section. Symbols: O, enzyme alone, with no addition of histone IIA or ATP and without 3-(3-dimethylaminopropyl)-1-ethylcarbodi-imide in the second incubation mixture; ●, no addition of histone or ATP in the first incubation, but 3-(3-dimethylaminopropyl)-1-ethylcarbodi-imide was added in the second incubation mixture; ■, 30 μ g of histone IIA was added to the first incubation mixture and 3-(3-dimethylaminopropyl)-1-ethylcarbodi-imide was added in the second incubation mixture; □, 1.5×10^{-9} mol (19 μ M) of ATP was added to the first incubation mixture and 3-(3-dimethylaminopropyl)-1-ethylcarbodi-imide was added in the second incubation mixture.

differ from the corresponding activities that appeared in the second curve from the bottom in both Figs. 1(a) and 1(b).

Effect of polyarginine on the inactivation of kinase by 3-(3-dimethylaminopropyl)-1-ethylcarbodi-imide

Since polyarginine competes with the protein substrate for the arginine-recognition site (Matsuo *et al.*, 1978a), modification and inactivations of the catalytic subunit were performed in the presence of a low concentration of polyarginine. Fig. 3 shows that inactivation of the enzyme is partially prevented by preincubation at 0°C with the competitive inhibitor. At higher concentrations, however, the enzyme activity will also be inhibited by polyarginine (Matsuo *et al.*, 1978b).

Fig. 3. Effect of polyarginine on the inactivation by carbodi-imide

Catalytic subunit (1.48 μ g) was preincubated with polyarginine (1.12 μ g) in a total volume of 25 μ l at 0°C for 10 min. Then 25 μ l of a solution containing 100 mM-Mes, pH 6.5, 200 mM-3-(3-dimethylaminopropyl)-1-ethylcarbodi-imide, but no glycine ethyl ester was added to the preincubation mixture. The mixture was then incubated at room temperature. At designated times, a portion (2 μ l) was removed and assayed for its protein kinase activity as described previously. Symbols: O, enzyme alone, but no polyarginine and no 3-(3-dimethylaminopropyl)-1-ethylcarbodi-imide added; ●, no polyarginine added in the preincubation mixture, but 3-(3-dimethylaminopropyl)-1-ethylcarbodi-imide was present in the second incubation mixture; ■, polyarginine was added in the first incubation mixture and 3-(3-dimethylaminopropyl)-1-ethylcarbodi-imide was present in the second incubation mixture.

Incorporation of [1-¹⁴C]glycine ethyl ester in the presence of 3-(3-dimethylaminopropyl)-1-ethylcarbodi-imide

If an appropriate nucleophilic reagent is used after the selective modification of carboxy groups in the proteins by carbodi-imide derivatives, the nucleophilic reagent can attack the activated carboxy group stoichiometrically to yield stable products (Hoare & Koshland, 1966). This carbodi-imide-nucleophile approach has been successfully used for the quantitative determination of carboxy groups in enzyme activity studies (Carraway & Koshland, 1972; Ramachandran & Colman, 1977; Pho *et al.*, 1977). We have used [1-¹⁴C]glycine ethyl ester as the nucleophilic reagent; the amount of [1-¹⁴C]glycine ethyl ester incorporated into the catalytic subunit of cyclic AMP-dependent protein kinase on a mol/mol basis was determined. Fig. 4 shows the extent of the labelled glycine ethyl ester incorporation versus the relative enzyme activity of protein kinase obtained at various incubation times

Fig. 4. Correlation between $[1-^{14}\text{C}]$ glycine incorporation and protein kinase inactivation

Catalytic subunit ($30.8\mu\text{g}$) was incubated with 3-(3-dimethylaminopropyl)-1-ethylcarbodi-imide and $[1-^{14}\text{C}]$ glycine ethyl ester in a total volume of $600\mu\text{l}$ under the conditions described in the Experimental section. At designated times, two portions were removed ($2\mu\text{l}$ and $25\mu\text{l}$ respectively). One portion ($2\mu\text{l}$) was used for the determination of protein kinase activity; the other was used for determining the amount of incorporated $[1-^{14}\text{C}]$ glycine methyl ester as described in the Experimental section.

with a fixed initial concentration ($67\mu\text{g/ml}$) of the enzyme. Our results indicate that at the maximum inactivation at the catalytic subunit 1.7 ± 0.2 mol of $[1-^{14}\text{C}]$ glycine ethyl ester was incorporated into one mol of catalytic subunit, implying the participation of 1.7 ± 0.2 carboxy groups in the function of protein kinase. The value of 1.7 is calculated on the basis of a molecular weight of 41000 for the catalytic subunit of cyclic AMP-dependent protein kinase (Matsuo *et al.*, 1978b).

Identification of amino acid residues modified by 3-(3-dimethylaminopropyl)-1-ethylcarbodi-imide and glycine ethyl ester

Since we have established that the loss of kinase catalytic activity by carbodi-imides in the presence of glycine ethyl esters was most probably due to carboxy group modification, attempts were made to determine which acidic amino acids, aspartate or glutamate, in the enzyme contained the functional carboxy group. The technique used by Ramachandran & Colman (1977), which involved the extensive proteolytic digestion of the inactivated enzyme modified by carbodi-imides and $[1-^{14}\text{C}]$ glycine ethyl esters followed by two successive high-voltage electrophoreses of the hydrolysate, was employed. Fig. 5(a) shows the result of the first high-voltage electrophoresis carried out at pH 6.4.

Fig. 5. Electrophoretograms of ^{14}C -labelled peptides from the catalytic subunit of cyclic AMP-dependent protein kinase

(a) Shows the high-voltage electrophoretic pattern of the proteolytically digested product of the ^{14}C -labelled catalytic subunit. Catalytic subunit ($430\mu\text{g}$) was incubated with 3-(3-dimethylaminopropyl)-1-ethylcarbodi-imide and $[1-^{14}\text{C}]$ glycine ethyl ester in a total volume of 1.5ml and then subjected to proteolytic digestion as described in the Experimental section. The hydrolysate was then subjected to high-voltage electrophoresis at pH 6.4 for 55 min at 2.5kV with β -aspartylglycine and γ -glutamylglycine as standards by the procedure of Ramachandran & Colman (1977). The paper strip was first stained with ninhydrin and then cut into segments ($2\text{cm} \times 1\text{cm}$). The radioactivity of these segments was determined individually. The histogram shows the radioactivity pattern of the electrophoretogram, and below this is shown the location of the standards in the same electrophoretogram. (b) Shows the identification of peak I material by high-voltage electrophoresis at pH 3.5. The material corresponding to the radioactive peak in the electrophoretogram in (a) was eluted from the unstained portion of the paper and subjected to high-voltage electrophoresis at pH 3.5 for 2 h at 2.5kV , also by the method of Ramachandran & Colman (1977). Again, the paper was stained with ninhydrin and its radioactivity was analysed. The histogram shows the radioactivity pattern and below this is shown the locations of the standards.

Only one region with marked radioactivity was observed; it co-migrated electrophoretically with the standard dipeptides of γ -glutamylglycine and β -aspartylglycine (Fig. 5a). After elution of the radioactive peak from the electrophoretic paper with water, it was further subjected to high-voltage electrophoresis at pH 3.5. As shown in Fig. 5(b), the electrophoretic position of the radioactive peak corresponded primarily to that of the standard dipeptide of γ -glutamylglycine, indicating that the glutamate residue is modified by the carbodi-imide-nucleophile reagents.

Discussion

Cyclic AMP-dependent protein kinase in mammalian skeletal muscle is a tetrameric enzyme, consisting of two pairs of dissimilar subunits called the catalytic and the regulatory subunits. The catalytic subunit catalyses the phosphorylation of phosphorylase kinase, glycogen synthase and phosphoprotein phosphatase III-inhibitor I (Nimmo & Cohen, 1977), whereas the regulatory subunit in the form of regulatory subunit-cyclic AMP complexes appears to inhibit the dephosphorylation of phosphorylase *a* (Gergely & Bot, 1978). Despite the important role of cyclic AMP-dependent protein kinases in the hormonal regulation of glycogen metabolism via the phosphorylation and the dephosphorylation mechanism, few attempts have been made to determine those amino acid residues that are critical for the function of cyclic AMP-dependent protein kinase. The present paper reports chemical evidence indicating the importance of carboxy groups of glutamate residues in the function of the catalytic subunit of cyclic AMP-dependent protein kinase from rabbit skeletal muscle.

The evidence for essential carboxy-containing residues in kinase activity is presented in Fig. 1. Carbodi-imides are known to modify carboxy groups, but other side reactions may also proceed under some conditions (Carraway & Koshland, 1972). In the present case, carbodi-imide inactivation of protein kinase does not occur at alkaline pH, and the inactivation at mild acidic pH follows a simple exponential function. Moreover, the total number of thiol groups of the catalytic subunit remains unchanged during the time course of the 3-(3-dimethylaminopropyl)-1-ethylcarbodi-imide modification reaction at pH 6.5. The fact that the inactivation does not occur at alkaline pH and that the total number of thiol groups does not change during the inactivation reaction can be taken as strong evidence that the loss of activity can be attributed primarily to carboxy-group modification by the carbodi-imide-nucleophile procedure of Carraway & Koshland (1972). In fact, high-voltage electrophoresis experiments carried out with peptide

fragments containing covalently bound label indeed show that the carboxy group of the glutamate residue is modified by carbodi-imide (Fig. 6).

The site of carbodi-imide modification is most likely located at the arginine-recognition site of the enzyme active centre based on the following lines of reasoning. (1) The loss of catalytic activity of the enzyme correlates well with the modification reaction as a function of time (Fig. 1), and, in addition, the inactivation is paralleled by a simultaneous incorporation of [^{14}C]glycine ethyl ester into the catalytic subunit of protein kinase (Fig. 4), leading to presumptive evidence that the carboxy group is a component of the enzyme active centre involved in the binding of substrate. (2) The enzyme can be protected from inactivation by histone, a protein substrate of the enzyme (Fig. 2). This protection may be explained in terms of a direct participation of the carboxy group as a component of the active centre in the binding of protein substrate. (3) ATP is another substrate of the enzyme; however, it does not protect the enzyme from inactivation by carbodi-imide-nucleophiles (Fig. 2). The results suggest that the carboxy group is not a component of the ATP-binding site in the enzyme active centre. This conclusion is consistent with the model proposed by Severin *et al.* (1978) that the ATP-binding site is composed primarily of an aromatic amino acid, a lysine and an arginine residue. In addition, it is also suggested that a nearby histidine residue is essential for the phosphotransferase reaction (Severin *et al.*, 1978). Since the carboxy group is a component of the enzyme active centre, but not a component of the phosphorylation site, and since the enzyme active centre can be considered to consist of an arginine-recognition site and a phosphorylation site (Matsuo *et al.* 1978b), the essential carboxy group can thus be reasonably taken as a component of the arginine-recognition site. (4) Polyarginine, a competitive inhibitor of the protein kinase, which is known from kinetic studies to interact specifically with the arginine-recognition site, protects the enzyme at low concentration (Fig. 3). Results of this experiment when considered with the effects of ATP and histone given above point to the carboxy groups of the arginine-recognition site as the most likely possibility for chemical modification. (5) As discussed in the introduction, the ionized carboxy group may form a bifurcated hydrogen-bond to two hydrogen atoms of the guanidinium group with the coulombic energy of the ion pairs as an additional stabilization force (Cotton *et al.*, 1971); hence, such a particularly good interaction may reasonably be expected to occur between the protein substrate having the general structure of -Arg-X-Y-Ser- and the carboxy-containing residues of protein kinase that are located at the arginine-recognition site.

It is noteworthy that the total loss of the kinase

activity during the time course of our studies corresponds to a modification of only 1.7 ± 0.2 mol of carboxy groups per mol of catalytic subunit (Fig. 4). It has been reported by Bechtel *et al.* (1977) that there are a total of 79 acidic and 57 basic amino acid residues in a catalytic subunit as revealed by amino acid analysis. These authors also reported that the purified catalytic subunit has activity corresponding to isoelectric points of pH 7.4 and 8.5 as determined by isoelectric focusing experiments. The presence of two isoelectric forms may represent the phosphorylated and non-phosphorylated protein kinase catalytic subunit. At pH 7.4, an isoelectric point of one form of the catalytic subunit, the protein should have no net electric charge. Thus the electric charge difference between the 79 acidic residues and 53 positively charged basic residues [34 Lys + 16 Arg + ($\frac{1}{2}$) $\times$ 7 His, by assuming half of the histidine residues being ionized at pH 7.4] must be due to the charge groups generated by the acid hydrolysis of asparagine and glutamine residues. This calculation, albeit crude, indicates that in the catalytic subunit there are about 53 residues having carboxy groups; it also raises the interesting question of why only about two out of the total 53 carboxy groups are detectable by the carbodi-imide-nucleophile reagents at pH 6.5 during the time course of the activity-modification studies (Figs. 4 and 5). Before we offer our explanations, it should be mentioned that carbodi-imides have been used, under similar conditions and pH, to inactivate carboxypeptidase A (Nau & Riordan, 1975), hexokinase (Pho *et al.*, 1977) and isocitrate dehydrogenase (Ramachandran & Colman, 1977). Results from these studies indicate that only one or two essential acidic residues per subunit of each enzyme are modified. Our result of 1.7 ± 0.2 carboxy groups per catalytic subunit is thus not at all surprising.

A simple explanation for our results is that the two carboxy groups in the arginine-recognition site may have relatively high pK_a values. The unusual pK_a may arise from a low dielectric constant in the local environment due to a multiple hydrophobic interaction of other nearby side chains. If this is the case, then these two carboxy groups are largely protonated at pH 6.5 owing to their unusually high pK_a , whereas other carboxy groups that are not involved in the protein substrate recognition or binding are ionized. It is the protonated carboxy groups that interact with the carbodi-imide (Kurzer & Douraghi-Zaceh, 1967); hence, only these two carboxy groups in the arginine-recognition site can be labelled with [1- 14 C]glycine ethyl esters in the presence of 3-(3-dimethylaminopropyl)-1-ethylcarbodi-imide at pH 6.5 during the time course of our studies. It should be emphasized, however, that, despite the unusual pK_a value, these two carboxy groups in the arginine-recognition site, may, if not

modified, still be largely in the ionized form at the assay or physiological pH values.

In summary, we have assumed that the active centre of the catalytic subunit of cyclic AMP-dependent protein kinase comprises both the arginine-recognition site and the catalytic site on the basis of our previous kinetic and structural studies (Matsuo *et al.*, 1978b). The present study provides strong evidence to suggest that two carboxy groups, most likely located at the arginine-recognition site, play a key role in the function of the enzyme. These two essential carboxy groups have been further identified as the γ -carboxy group of the glutamate residue. The precise role of these residues in recognizing the protein substrate with a local primary sequence -Arg-X-Y-Ser- remains to be elucidated. However, because of the possibility that the essential carboxy groups may have relatively high pK_a values, we suggest that a perfect complementary arginine-recognition site may not exist totally as such until the positively charged arginine residue in the primary structure -Arg-X-Y-Ser- of the protein substrate arrives at the locality to dissociate completely the essential carboxy groups. Once the complementary recognition is achieved, the binding may be accompanied by structural orientations in the enzyme as well as the protein substrate so that the serine residue in the substrate sequence -Arg-X-Y-Ser- can be phosphorylated by the catalytic site of the active centre.

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