from pyrophosphate exchange and pre-steady-state kinetics have to be reviewed. In particular, the calculations of the free energy of formation of aminoacyl adenylates are presumably incorrect as additional binding constants are involved.

References

Blanquet, S., Fayat, G., Waller, J.-P., and Iwatsubo, M. (1972), *Eur. J. Biochem. 24,* 461.
Bosshard, H. R., Koch, G. L. E., and Hartley, B. S. (1974), submitted for publication.
Eigen, M., and Hammes, G. G. (1963), *Advan. Enzymol. Relat. Areas Mol. Biol. 24,* 13.
Englund, P. T., Huberman, J. A., Jovin, T. M., and Kornberg, A. (1969), *J. Biol. Chem. 244,* 3038.
Fersht, A. R. (1975), *Biochemistry 14,* 5.
Fersht, A. R., Ashford, J. S., Bruton, C. J., Jakes, R., Koch, G. L. E., and Hartley, B. S. (1975), *Biochemistry 14,* 1.
Fersht, A. R., and Renard, M. (1974), *Biochemistry 13,* 1416.
Hélène, C., Brun, F., and Yaniv, M. (1971), *J. Mol. Biol.* 58, 349.
Hill, A. V. (1910), *J. Physiol. (London) 40,* iv.
Holler, E., and Calvin, M. (1972), *Biochemistry 11,* 3741.
Hummel, J. P., and Dreyer, W. J. (1962), *Biochim. Biophys. Acta 63,* 530.
Loftfield, R. B. (1972), *Progr. Nucl. Acid Res. Mol. Biol. 12,* 87.
Renard, M., and Fersht, A. R. (1973), *Biochemistry 12,* 4713.
Yarus, M., and Berg, P. (1969), *J. Mol. Biol. 42,* 171.

Rabbit Skeletal Muscle Protein Kinase. Conversion from cAMP Dependent to Independent Form by Chemical Perturbations†

Laura C. Huang and Ching-hsien Huang*

ABSTRACT: Protein kinase isolated from rabbit skeletal muscle can be reversibly converted from the cAMP dependent form to the independent form by chaotropic salts and urea. A similar but irreversible conversion can also be induced by trypsin digestion of the holoenzyme. The dissociation of cAMP dependent protein kinase by low concentrations of thiocyanate raises the possibility of isolating both *native* regulatory and catalytic subunits. From various changes in enzymatic activity caused by urea and trypsin perturbation, it is proposed that the conversion of protein kinase from the cAMP dependent to the independent form is due primarily to preferential modification of the regulatory subunit of the holoenzyme.

Cyclic adenosine 3′,5′-monophosphate dependent protein kinases have been a subject of great interest in recent years (see Langan (1973); Walsh and Ashby (1973) for reviews). Several independent groups (Brostrom *et al.,* 1970; Gill and Garren, 1970; Kumon *et al.,* 1970; Tao *et al.,* 1970) have proposed the "dissociation model" to explain the mode of action of cyclic adenosine 3′,5′-monophosphate (cAMP)[1] on the protein kinase. In this model, cAMP promotes the dissociation of inactive holoenzyme to yield a regulatory subunit–cAMP complex and a free, active catalytic subunit. This dissociated catalytic subunit behaves as a cAMP independent protein kinase. Based on the simple "dissociation model," one would expect that agents which facilitate the dissociation would activate the cAMP dependent protein kinase, thus converting the protein kinase from a cAMP dependent form to a cAMP independent form.

Recently, it has been reported that chaotropic salts can induce dissociation of proteins such as β-lactoglobulin A, concanavalin A, and hemoglobin at relatively low concentrations without causing major shifts in protein conformation (Sawyer and Puckridge, 1973). In addition, it has been observed that potassium thiocyanate promotes dissociation of glutamine-dependent carbamyl phosphate synthetase into catalytically dissimilar subunits (Trotta *et al.,* 1974). These studies led us to investigate the proposal that the catalytic subunit of the protein kinase may be released from the inhibitory binding subunit upon the addition of chaotropic salts and thus make the catalytically active subunit available for phosphorylation in the absence of cAMP. In addition, other chemical agents such as urea and trypsin are well-known for their effects on the structural properties of water-soluble enzymes. They may also shift the equilibrium of the holoenzyme toward the dissociation and, accordingly, convert the protein kinase from a cAMP dependent form to a cAMP independent form.

The objectives of the present study were twofold: (1) to isolate purified cAMP dependent protein kinase from rabbit skeletal muscle and (2) to convert the enzyme from its cAMP dependent form to its independent form by chemical perturbation. From these studies, it is suggested that modification of the regulatory subunit is of primary importance in activating cAMP dependent protein kinase.

† From the Departments of Pharmacology (L.C.H.) and Biochemistry (C.H.), University of Virginia School of Medicine, Charlottesville, Virginia 22901. *Received August 22, 1974.* This research was supported in part by National Science Foundation Grant GB-38432 and U. S. Public Health Service Grant GM-17452.

[1] Abbreviations used are: cAMP, cyclic adenosine 3′,5′-monophosphate; DTT, dithiothreitol (Cleland's reagent).

Experimental Section

Materials. $^{32}P_i$ was purchased from New England Nuclear and used to prepare [γ-^{32}P]ATP by the method of Glynn and Chappell (1964). DEAE-cellulose was obtained from Gallard-Schlesinger. Sephadex G-200 was supplied by Pharmacia. DEAE-Bio-Gel, HTP-hydroxylapatite, acrylamide, and other reagents for gel electrophoresis were purchased from Bio-Rad Laboratories. Ultra-pure urea and enzyme-grade ammonium sulfate were obtained from Schwarz/Mann. Dithiothreitol (DTT) was purchased from Calbiochem. Phenylmethanesulfonyl fluoride and histone IIA were obtained from Sigma. Trypsin and tryspsin inhibitor were supplied by Worthington. Other enzymes and proteins used for molecular weight determinations were obtained, according to availability, from Calbiochem, Sigma, or Worthington. All other chemicals were of reagent or analytical grade. Water was deionized and glass-distilled.

Assay of Protein Kinase. Protein kinase activity was measured as the conversion of radioactivity labeled [γ-^{32}P]ATP into histone as described previously (Huang, 1974) except for the use of histone IIA in place of histone IV. The composition of the preincubation medium varied with the experiment and is stated in each case in the relevant figure legend. Protein concentration was estimated by the method of Lowry *et al.* (1951).

Preparation of Rabbit Skeletal Muscle cAMP Dependent Protein Kinase. The purification of rabbit muscle cAMP dependent protein kinase briefly described below is a modification of that described by Yamamura *et al.* (1973). All steps were conducted in a cold room at 4°. Fresh rabbit muscle (400 g) was homogenized with 2.5 volumes of chilled 0.01 M potassium phosphate buffer (pH 7.5) containing 6 mM 2-mercaptoethanol, 5 mM EDTA, 2 mM phenylmethanesulfonyl fluoride, and 10% glycerol (v/v). After centrifugation of the homogenate at 20,000*g* for 20 min, solid ammonium sulfate was added into the supernatant to a final concentration of 60%. The ammonium sulfate mixture was centrifuged again, and the resultant precipitate was dissolved in a minimum volume of Tris-HCl buffer (0.01 M) (pH 7.5) containing 6 mM 2 mercaptoethanol, 5 mM EDTA, 2 mM phenylmethanesulfonyl fluoride, and 10% glycerol. After dialyzing against the same Tris buffer, the enzyme preparation was applied to a DEAE-cellulose ion exchange column (4 × 34 cm) which had been previously washed and equilibrated with 0.01 M Tris-HCl buffer (pH 7.5) containing 6 mM 2-mercaptoethanol, 2 mM EDTA, and 10% glycerol. After sample loading, the column was then eluted with 0.03 M potassium phosphate buffer (pH 7.0) containing 2 mM EDTA and 6 mM 2-mercaptoethanol. Enzymatic activity was determined in the eluted fractions. Those fractions which contain cAMP dependent protein kinase were pooled and precipitated by addition of solid ammonium sulfate to 40% of saturation. The precipitate was dissolved in a small volume of 0.01 M Tris-HCl buffer (pH 7.5) containing 2 mM EDTA and 1 mM DTT, and the dissolved sample was thoroughly dialyzed against the same buffer. The enzyme solution was chromatographed on a DEAE-Bio-Gel column. Prior to use, the DEAE-Bio-Gel had been washed with 0.01 M Tris-HCl buffer (pH 7.5) containing 2 mM EDTA and 1 mM DTT. After the enzyme solution entered the gel, it was eluted with a positive linear NaCl gradient (0.05–0.40 M) in the same buffer solvent. Only one major peak of enzymatic activity was detected in the eluate which was directly applied to a hydroxylapatite column (2.5 × 5 cm). The hydroxylapatite column, which had been previously washed with 50 ml of 0.03 M phosphate buffer (pH 7.0) containing 1 mM DTT, was stepwise eluted with 100 ml of 0.03, 0.06, 0.15, and 0.30 M phosphate buffer (pH 7.0) each containing 1 mM DTT. The protein kinase activity was found to be eluted with both 0.15 and 0.30 M phosphate buffer fractions. Since enzyme in the 0.15 M fractions is greatly stimulated by cAMP whereas that in the 0.30 M fractions is cAMP independent, only the cAMP stimulated enzyme was precipitated from the 0.15 M phosphate fractions after centrifugation by addition of ammonium sulfate to 40% of saturation. The precipitate was dissolved in a small volume of 0.1 M NaCl solution containing 0.01 M phosphate at pH 7.0, 1 mM DTT, 2 mM EDTA, and 10% glycerol, and the resultant sample was then subjected to gel filtration on a preparative Sephadex G-200 column (2.5 × 45 cm) equilibrated with the same buffered NaCl–glycerol solution. After determining their activities and absorbances, fractions were frozen and stored at −40° until required.

Isoelectrofocusing. This was performed in a linear sucrose or glycerol gradient containing 2% ampholine (LKB), pH 5–8, in the LKB Model 8101 column (110-ml capacity) according to Vesterberg (1971). Electrofocusing was carried out at 4° for 28 hr with a final voltage of 600 V. Fractions of 15 drops were collected and their pH was measured at room temperature with a Radiometer PHM 4. cAMP dependent protein kinase activity in each fraction was determined after diluting the sample twofold with 0.1 M glycylglycine buffer at pH 7.0.

Polyacrylamide Gel Electrophoresis. Sodium dodecyl sulfate polyacrylamide gel electrophoresis was carried out on 5.3% polyacrylamide gels containing 0.1% sodium dodecyl sulfate following the procedure of Weber and Osborn (1969). Densitometric tracings of Coomassie Blue stained gels were performed on a Gilford recording spectrophotometer equipped with a gel scanner. Molecular weights of protein kinase components were estimated from relative mobility of appropriate molecular weight markers on sodium dodecyl sulfate gels.

Polyacrylamide disc gel electrophoresis of the native enzyme was performed according to the method of Davis (1964), except that the stacking gel was usually omitted. The 5.3% polyacrylamide separation gel had a pH of either 9.3 or 4.3. After electrophoresis, the gel was immersed in 5% trichloroacetic acid for 15 min before staining with Coomassie Blue. When the enzymatic activity was to be located in the gels after electrophoresis, the gels were first immersed in 0.1 M phosphate buffer (pH 7.0) for 40 min, and then cut into small segments with a Bio-Rad gel slicer. The small segments were placed separately into test tubes containing assay buffer, and the assay was performed in the usual manner.

Analytical Molecular Sieve Chromatography. The experiment was carried out with a standard Pharmacia jacketed column (1.6 × 75 cm) of Sephadex G-200 with flow adaptors that had been equilibrated with 1 l. of 0.1 M NaCl in 0.01 M potassium phosphate buffer (pH 7.0) containing 2 mM EDTA and 1 mM DTT. The column was run upward flow and the column effluent was connected to a Zeiss MZ2D microflow cell. The outflow tube of the microflow cell was connected to a peristaltic pump (Holter pump, Model RL175) which could be adjusted to give the desired flow rate (5 ml/hr). Aliquots of 1 ml each were collected successively in a fraction collector. Protein markers of approximately 0.5–1 mg each were dissolved in a volume of

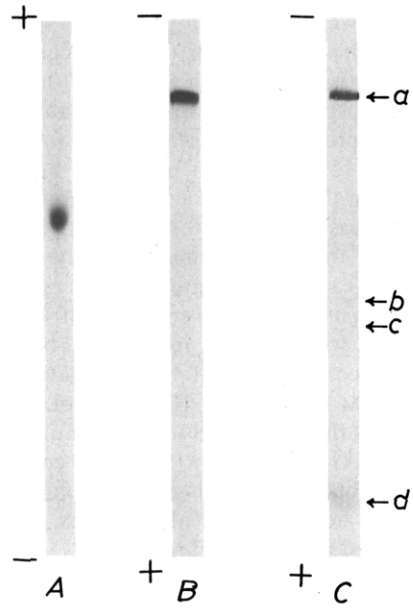

FIGURE 1: Polyacrylamide gel electrophoresis. (A) Native cAMP dependent protein kinase was run in a 5.3% gel at pH 4.3; (B) native cAMP dependent protein kinase was run in a 5.3% gel at pH 9.3; (C) the sodium dodecyl sulfate dissociated enzyme was run in a 5.3% gel that contained 0.1% sodium dodecyl sulfate. The heavy major band, a, corresponding to a molecular weight of about 180,000 and two faint polypeptide components, b and c, with molecular weight of about 48,000 and 42,000, respectively, are indicated. An internal marker, cytochrome *c*, was run together with the sodium dodecyl sulfate treated sample, and it migrated electrophoretically ahead of the band of Bromophenol Blue tracking dye as shown by the band d.

300 μl of equilibration buffer and the solution was subjected to upward flow gel filtration at 4°. The column effluent was continuously monitored at 230 nm by a Zeiss PMQ II spectrophotometer equipped with the automatic sample changer and a multipoint recorder. Elution volume, V_e, of the marker was detected by absorbance at 230 nm in the recorded elution profile. The excluded volume of Blue Dextran was taken as the void volume, V_0, of the column. Dinitrophenylalanine was used to determine the internal volume, V_i, as detected by absorbance at 360 nm. Samples of protein kinase were assayed for enzymatic activity in the presence of cAMP. The aliquot having the maximum enzymatic activity was taken as the elution volume of cAMP dependent protein kinase. Partition coefficients of protein markers and of protein kinase were calculated from the equation $K_d = (V_e - V_0)/(V_i - V_0)$, and the molecular weight of cAMP dependent protein kinase was estimated from the linear relationship between K_d and log (molecular weight) for protein markers (Laurent and Killander, 1964). The inverse error function complement of the partition coefficient ($\text{erfc}^{-1} K_d$) was used to estimate both the diffusion coefficient and Stokes radius of the enzyme (Ackers, 1970).

Similar ascending gel filtration experiment was carried out at 4° in a standard Pharmacia K16/75 jacketed column packed with Sephadex G-200 that had been equilibrated and was eluted with 0.3 M NaSCN in 0.01 M potassium phosphate buffer (pH 7.0) containing 2 mM EDTA and 1 mM DTT. Because of the high background absorbance, the column effluent was recorded at 280 nm. Protein markers of approximately 1–3 mg each in a volume of 300 μl were used, and their elution volumes were detected by absorbance at 280 nm.

Results

General Characteristics of cAMP Dependent Protein Kinase. Purification of cAMP dependent protein kinase by the procedures outlined earlier resulted in essentially pure protein when analyzed on polyacrylamide gels (Figure 1A and B). That this single protein band was protein kinase was substantiated in a duplicate, unstained gel by determining the enzyme activity and demonstrating that the enzyme activity had the same location as the protein band stained with Coomassie Blue.

The average p*I* of cAMP dependent protein kinase was 5.27 as determined by its maximum activity position obtained from two separate electrofocusing experiments. One was carried out in a sucrose gradient (p*I* = 5.30), while the other was performed in a glycerol gradient (p*I* = 5.24). This mean value of p*I* is in excellent agreement with the literature value of 5.2 (Yamamura *et al.*, 1973).

The polypeptide banding patterns resulting from sodium dodecyl sulfate polyacrylamide gel electrophoresis were complex. One major component corresponding to a species with an apparent molecular weight of 180,000 and two minor stained bands of 48,000 and 42,000 were generally observed (Figure 1C). The major component of 180,000 was not an artifact of the electrophoretic procedure, since the same band can be demonstrated using either gels containing 1% sodium dodecyl sulfate or carboxymethylated enzyme sample. The relationship between the holoenzyme and these polypeptide chains observed with sodium dodecyl sulfate gels is currently under investigation.

Estimation of Molecular Weight, Stokes Radius, and Diffusion Coefficient of cAMP Dependent Protein Kinase by Analytical Molecular Sieve Chromatography. From the gel filtration behavior of the following standard protein markers: rabbit pyruvate kinase (235,000), yeast alcohol dehydrogenase (150,000), rabbit enolase (82,000), ovalbumin (45,000), and chymotrypsinogen A (25,000), an apparent molecular weight of cAMP dependent protein kinase was estimated. This value, 226,000 ± 5%, was obtained based on the partition coefficient, 0.180, of the enzyme eluted with 10 mM phosphate buffer (pH 7.0) containing 0.1 M NaCl–2 mM EDTA–1 mM DTT.

The correlation between the inverse error function complement of the partition coefficient ($\text{erfc}^{-1} K_d$), Stokes radius (R_s), and reciprocal of diffusion coefficient (D) for analytical molecular sieve chromatography of standard protein markers and cAMP dependent protein kinase was also plotted. The molecular parameters of the protein markers were taken mainly from Andrews (1970), except those of enolase were taken from Winstead and Wold (1965). The diffusion coefficient, 4.06×10^{-7} cm^2/sec, and Stokes radius, 52.5 Å, of the enzyme were found to be very close to the corresponding values for pyruvate kinase. Here, we should emphasize that the elution volume of cAMP dependent protein kinase was determined by measuring the enzymatic activity of each individual fraction of the column effluent. The protein peak which was barely detected by absorbance at 230 nm, however, eluted slightly behind the activity peak, indicating that the enzyme may not be 100% pure.

Effects of Chaotropic Salts on cAMP Dependent Protein Kinase. The comparative effects of NaSCN and KSCN on the protein kinase activity assayed with and without cAMP are shown in Figure 2. With increasing thiocyanate concentration, the enzymatic activity assayed in the presence of cAMP shows a slow reduction in phosphorylation, and the activity is seen to reach 64% of its initial value at 0.3 M

chaotropic salt. The fact that NaSCN and KSCN affect the activity identically within the concentration range studied suggests that thiocyanate anions, not the cations, are contributing to the decrease in enzymatic activity. In contrast, the protein kinase activity assayed in the absence of cAMP shows that the added chaotropic salts activate the enzyme and the maximum activation is observed between 0.2 and 0.3 M. It is worthwhile to point out that within the concentration range of 0.3–0.5 M of thiocyanate, the enzyme is completely converted from the cAMP dependent form to the cAMP independent form as shown by the essentially identical activity curves. The increase in enzymatic activity of protein kinase assayed in the absence of cAMP upon addition of chaotropic salts can be ascribed to the dissociation of the catalytic subunit from the inhibitory subunit, since it is known that thiocyanates promote the dissociation of oligomeric enzymes (Sawyer and Puckridge, 1973). The observation that the enzymatic activity increases to a maximum around 0.2–0.3 M thiocyanate and then falls off slowly at high concentrations can be explained as a net result of the following two opposite effects of the chaotropic salt: (1) it promotes the dissociation of catalytic subunits from inhibitory regulatory subunits and thus activates the enzyme, (2) it also directly inhibits, but to a lesser extent, the catalytic activity of the functional subunit.

The dissociation of cAMP dependent protein kinase by thiocyanate was substantiated by the gel filtration behavior of the enzyme. After dialyzing against 0.3 M NaSCN containing 10 mM phosphate buffer (pH 7.0), 2 mM EDTA, and 1 mM DTT, an aliquot of the NaSCN–enzyme solution was subjected to ascending molecular sieve chromatography on a Sephadex G-200 column equilibrated with the same buffered 0.3 M NaSCN solution.

The partition coefficient, K_d, of the protein kinase determined in the presence of 0.3 M NaSCN gave an enzymatically active unit of molecular weight about 40,000. In addition, the activity of the eluted enzyme was found, as expected, to be cAMP independent. These results clearly demonstrate that an active catalytic subunit of cAMP dependent protein kinase can be dissociated from the holoenzyme by NaSCN.

The conversion of protein kinase from the cAMP dependent to the independent form by thiocyanate can be further shown to be reversible. After prolonged dialysis against 0.3 M NaSCN, the enzyme was found to be less active and cAMP independent. The thiocyanate, however, could be removed from the 0.3 M NaSCN-inhibited enzyme by gel filtration on a Sephadex G-200 column equilibrated with 0.1 M NaCl–10 mM phosphate buffer–2 mM EDTA–1 mM DTT. It was found that the eluted enzyme appeared at approximately the same elution volume as the native holoenzyme. Moreover, the activity of the eluted thiocyanate-free enzyme was greatly stimulated by cAMP. These results show that the dissociation of cAMP dependent protein kinase which occurs in the presence of thiocyanate can be reversed by removing the dissociating reagent.

The results of the effects of NaCl and KCl on the cAMP dependent protein kinase activity indicate that these salts are also effective, but to a lesser degree than NaSCN or KSCN, in slowly blocking the activity of the enzyme as assayed in the presence of cAMP. Unlike the case of NaSCN and KSCN, within the concentration range (0.0–0.5 M) studied, NaCl and KCl do not stimulate appreciably the enzymatic activity of cAMP dependent protein kinase as assayed in the absence of cAMP. These results are consistent

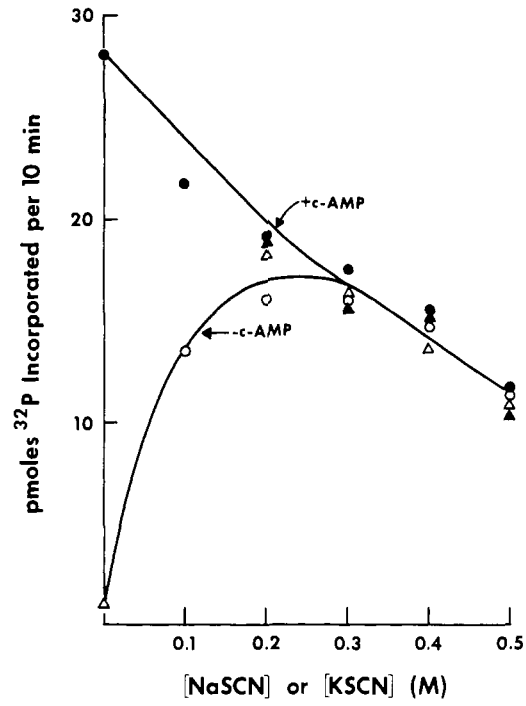

FIGURE 2: Effects of concentration of NaSCN and KSCN on the activity of rabbit skeletal muscle protein kinase assayed in the presence and absence of cAMP. An aliquot of purified protein kinase, 2.5 μg in 10 μl, was preincubated at 0° in 300 μl of 0.1 M glycylglycine buffer (pH 7.0) containing various concentrations, as indicated, of NaSCN and KSCN, respectively. After 1 hr of preincubation, 10 μl of the enzyme solution was pipetted into the assay mixture which contained 10 μmol of glycylglycine (pH 7.0), 0.3 μmol of $MgCl_2$, 0.5 mg of histone IIA, 1.8 nmol of [γ-^{32}P (190 cpm/pmol), and 0.1 nmol of cAMP if added, in a total volume of 90 μl. Protein kinase activity was expressed as pmole of ^{32}P incorporated per 10 min. (●) Activity obtained with KSCN as assayed in the presence of cAMP; (○) activity assayed in the absence of cAMP; (▲) activity obtained with NaSCN as assayed in the presence of cAMP; (△) results when assayed in the absence of cAMP.

with the reported order of effectiveness of these salts in causing dissociation of β-lactoglobulin A which followed the Hofmeister series: $CCl_3COO^- > SCN^- > CF_3COO^- > Br^- > NO_3^- > Cl^-$ (Sawyer and Puckridge, 1973).

Effect of Urea. The addition of urea to cAMP dependent protein kinase in aqueous solution, at 0°, up to 4.5 M caused no significant alteration in the enzymatic activity when assayed in the presence of cAMP. Further addition of urea, however, results in an abrupt reduction in enzymatic activity, which reaches nearly zero as the concentration of urea approaches 6 M. These results, shown in Figure 3, demonstrate that urea in a concentration up to 4.5 M has no measurable effect on the active catalytic subunit of cAMP dependent protein kinase and that complete inactivation of the catalytic subunit occurs at 6 M urea.

In the absence of cAMP, the enzymatic activity can be increased by the addition of urea, and the activation reaches a maximum at 4.5 M. Above 4.5 M urea, the activity curve falls off abruptly which, as shown in Figure 3, parallels the curve observed for the enzyme assayed in the presence of cAMP. It should be emphasized that the enzymatic activity of the protein kinase could be converted totally from the cAMP dependent form to the independent form upon addition of 4.0–4.5 M urea, provided that the enzyme was preincubated with the urea solution for 1 hr or longer (data not given). The 4.0 M urea-treated enzyme regained its cAMP dependency when the urea was removed by dialysis.

The conversion of protein kinase from a cAMP depen-

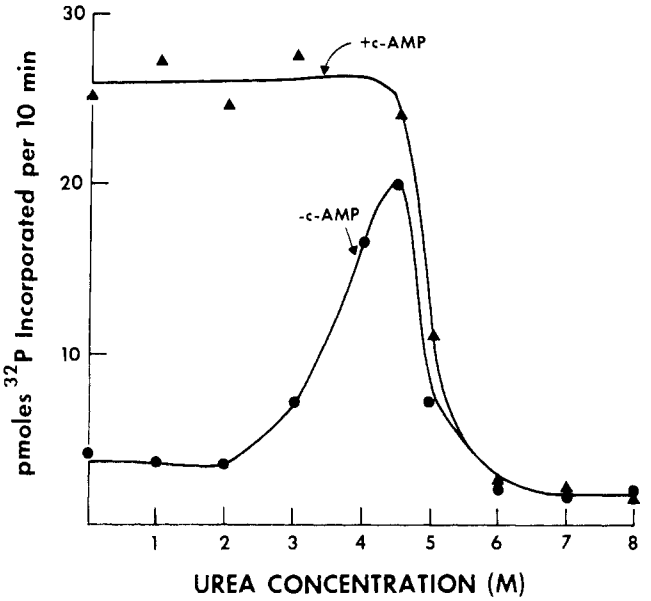

FIGURE 3: Effect of urea on the activity of protein kinase assayed in the presence (▲) and absence (△) of cAMP. Conditions are same as specified in Figure 2 except that the protein kinase was preincubated in various concentrations of urea as indicated in the figure for 10 min at 0°.

dent to a cAMP independent form upon addition of urea indicates that there is a differential effect of urea on the regulatory (or inhibitory) and catalytic subunits of the holoenzyme. Our data suggest that although urea up to 4.5 M exerts little effect on the catalytic subunit, it must have perturbed the regulatory subunit structure in such a way that the regulatory subunit can no longer be inhibitory toward the enzymatic activity of catalytic subunit. In other words, urea at lower concentrations preferentially denatured the regulatory subunits in the holoenzyme.

Trypsin Digestion of cAMP Dependent Protein Kinase. The change in activity of the tryptic digestion mixture with time, measured in the presence and absence of cAMP, is presented in Figure 4. With trypsin treatment, maximum activation of the enzyme as assayed in the absence of cAMP was reached in 10 min under these experimental conditions. Activity decreased slightly from 10 to 30 min. When assayed in the presence of cAMP, however, a significant, progressive decrease in enzymatic activity was observed over the time course recorded. When, prior to the addition of trypsin, trypsin inhibitor was added to the reaction mixture, enzymatic activities measured in the presence and absence of cAMP remained essentially constant, as indicated in Figure 4. Since trypsin digestion can generally modify the structure of an enzyme and thus lead to weakening of its subunit interactions, one would thus expect to see a change in enzymatic activity due to the tryptic attack. However, if the catalytic subunits and regulatory subunits of the holoenzyme were structurally arranged in such a way that they would be equally exposed to the tryptic digestion, then the positive activation effect on activity as caused by the selective modification of regulatory subunit structure would have cancelled the negative inactivation effect caused by the equally probable attack of catalytic subunits. One simple explanation for the observed increase in enzymatic activity as assayed in the absence of cAMP might be that the regulatory subunits are arranged on the outside of the catalytic subunits; this arrangement makes the regulatory subunit more susceptible to trypsin attack. The decrease in activity as assayed in the presence of cAMP, however, suggests that portions of the catalytic subunits are also susceptible to trypsin attack.

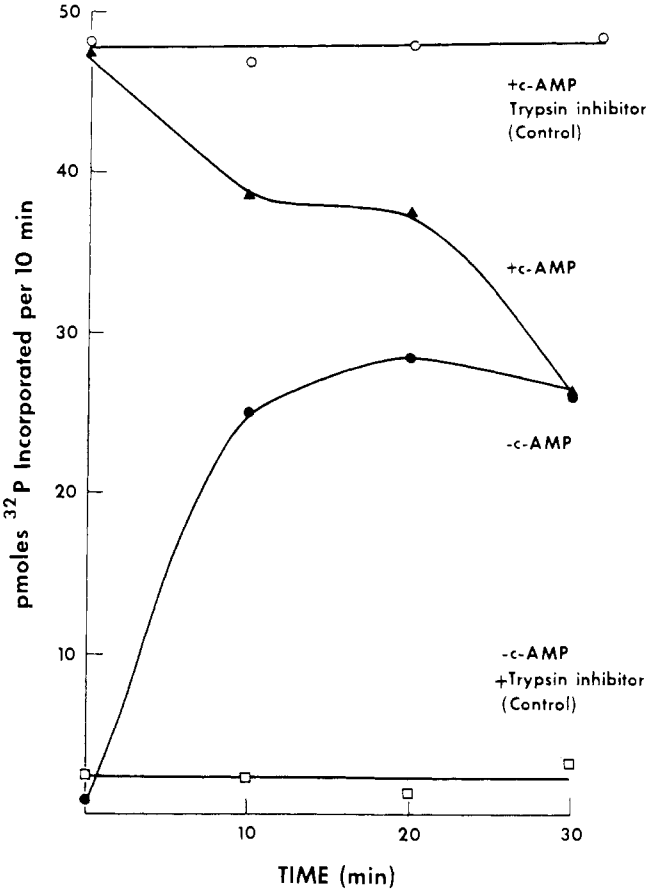

FIGURE 4: The change in enzymatic activity of rabbit skeletal muscle protein kinase after tryptic digestion. After various times, as indicated in the figure, of preincubation of the enzyme (75 μg in 100 μl of 0.1 M glycylglycine (pH 7.0)) with 5 μg of trypsin at 30°, the tryptic hydrolysis was terminated by mixing 10 μl of the enzyme–trypsin solution with 300 μl of 0.1 M glycylglycine buffer (pH 7.0) containing 50 μg of trypsin inhibitor. The protein kinase activity was measured in the presence and absence of cAMP under the conditions specified in Figure 3. In a parallel control experiment, 50 μg of trypsin inhibitor was added to the enzyme solution prior to the mixing of trypsin with the enzyme solution.

Discussion

Two different cAMP dependent protein kinases, B_1 and B_2, isolated from rabbit muscle have been described by Yamamura *et al.* (1973). The cAMP dependent protein kinase presented in this report corresponds closely to their B_1 kinase based on the following parallel lines of evidence: (1) in the step of hydroxylapatite column chromatography, both enzymes are eluted with 0.15 M phosphate, and (2) both enzymes have the identical isoelectric point of pH 5.2. The molecular parameters of the B_1 kinase have not yet been reported, hence our estimated values of molecular weight, 226,000, and Stokes radius, 52.5 Å, cannot be compared.

Corbin *et al.* (1972) using gel filtration and sucrose density gradient centrifugation estimated a molecular weight and Stokes radius of a cAMP dependent protein kinase isolated from rabbit skeletal muscle of 123,000 and 44 Å, respectively. Erlichman *et al.* (1973) reported that bovine heart cAMP dependent protein kinase had a molecular

weight of 175,000 using sedimentation equilibrium measurements and a Stokes radius of 60 Å obtained by gel filtration. Our value of 226,000 as the molecular weight for cAMP dependent protein kinase is considerably greater than the values reported by Corbin *et al.* (1972) and Erlichman *et al.* (1973), respectively. The Stokes radius of the cAMP dependent protein kinase under study, however, is markedly smaller than that reported for the bovine heart muscle enzyme. The discrepancy between our data and those estimated by Corbin *et al.* (1972) reflects one or more of the following possibilities: (1) The cAMP dependent protein kinase may exist in multiple forms in rabbit skeletal muscle, and it is possible that different groups were working on different forms of the enzyme. (2) There is only one common form of cAMP dependent protein kinase from rabbit skeletal muscle. The kinase may undergo various degrees of proteolysis during isolation, leading to kinases of different molecular dimensions when isolated. (3) The gel-filtration data from the present study for estimation of molecular weight could give an anomalous result.

It is well documented that a molecule of high axial ratio such as fibrinogen will generally appear to have a higher apparent molecular weight on gel filtration than its true molecular weight. Also, a molecule such as ferritin with an unusual value for its partial specific volume behaves anomalously during gel filtration (Andrews, 1970). Our value of 226,000 ± 5% for the molecular weight of cAMP dependent protein kinase was determined by gel filtration, and must be taken cautiously as a crude estimation, since both the shape and the partial specific volume of the enzyme are unknown. However, gel filtration provides an accurate means of studying molecular size (Ackers, 1970); hence our value of 52.5 Å calculated from the gel-filtration data represents a good approximation of the Stokes radius of cAMP dependent protein kinase.

The potential contamination of protein kinases by proteases during the isolation procedures has been previously discussed by Corbin *et al.* (1972) and Yamamura *et al.* (1973). Attempts were specifically made in this work to eliminate proteolysis and to thus preserve the physical integrity of the isolated enzymes. The protease inhibitor, phenylmethanesulfonyl fluoride, was first added to the enzyme mixture to control the action of protease during the initial few steps of purification. A low percentage of ammonium sulfate (40%) was added in the later steps to precipitate the relatively larger cAMP dependent protein kinase and thus separate it from proteases and other minor contaminants. Furthermore, with the exception of the hydroxylapatite column step, EDTA was used throughout the whole purification procedure to minimize the suspected proteolysis. Because these strict precautions were taken, it seems probable that the purified enzyme used in this study was not modified by proteolysis which may explain why we obtained a higher value for the Stokes radius than the value reported by Corbin *et al.* (1972).

Both the observed changes in the cAMP dependency of the enzymatic activity (Figure 2) and the observed increase in the partition coefficient of the enzyme in the presence of thiocyanate lend support to the proposal that the catalytic subunit of the protein kinase may be released from the regulatory subunit upon the addition of chaotropic salts. The dissociation induced by solvent perturbation may, therefore, provide a novel approach to the isolation of both native regulatory and catalytic subunits. Because of the extremely tight binding of cAMP, native regulatory subunits cannot

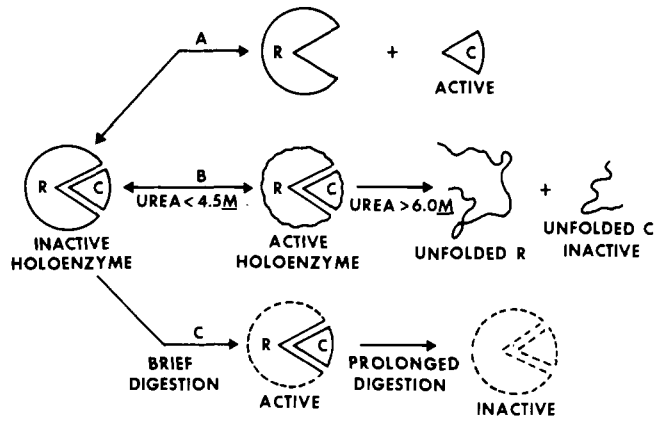

FIGURE 5: A schematic representation showing the possible mechanism of the activation of the cAMP dependent protein kinase by (A) chaotropic salts; (B) urea; and (C) trypsin. The regulatory subunit or *yin* of the holoenzyme is diagrammatically shown as R, while the catalytic subunit or *yang* is C.

be readily separated from the regulatory subunit–cAMP complex obtained by dissociation of protein kinase with cAMP (Brostrom *et al.*, 1971). In contrast, the chaotropic salts can be easily removed from the protein by dialysis or gel filtration. Experiments are now in progress to ascertain the possibility of isolating native regulatory and catalytic subunits from cAMP dependent protein kinase with chaotropic salts.

The "dissociation model" proposed for the action of cAMP on the protein kinase (Brostrom *et al.*, 1970) forms the basis for our discussions regarding the various effects of chaotropic salts, urea, and trypsin. In this model, the holoenzyme is essentially devoid of catalytic activity because of the inhibitory action of regulatory subunit. Protein kinase can, however, be dissociated into an active catalytic subunit and a cAMP–regulatory subunit complex in the presence of cAMP. Our data provide strong evidence that the cAMP dependent protein kinase can be converted to a cAMP independent kinase upon mild solvent and chemical perturbation. We propose that this conversion is likely due to a preferential effect of urea and trypsin on the regulatory subunit and further interpret this preferential effect as a consequence of asymmetric arrangement of the two subunits in the holoenzyme. The chaotropic salts, however, have a general dissociating effect on the holoenzyme. A schematic representation of the effects of various perturbing agents is shown in Figure 5. The regulatory subunit, R, is diagrammatically shown to be exposed to the surrounding medium more openly than the catalytic subunit, C, and thus is more susceptible to perturbation. Here, we emphasize the modification of the regulatory subunit by the solvent or chemical perturbation. The dissociation of the holoenzyme may simply be a natural consequence of the impaired cooperative interaction between two subunits as caused by the modification of R. It is possible, therefore, that protein kinase may become active and cAMP independent without dissociation, provided the structure of the regulatory subunit is so modified that its inhibitory effect can no longer be propagated through the subunit–subunit interacting domain to the catalytic subunit.

Studies on the effects of chaotropic salts, urea, and trypsin on the enzymatic activity of cAMP dependent protein kinase under present investigation provide only *presumptive* evidence that regulatory subunits of the inactive holoenzyme can be preferentially modified and, consequently, pro-

tein kinase can be converted from a cAMP dependent form to a cAMP independent form. Efforts are currently being made in this laboratory to scale up the yield of purified cAMP dependent protein kinase. Direct structural studies on the isolated protein kinase in the presence of these perturbing agents will be under investigation.

Acknowledgments

We are indebted to James P. Charlton and Howard C. Froehlich for their dedicated technical assistance in the laboratory. We also extend thanks to Robert G. Langdon, Joseph Larner, and Carter M. Anderson, for comments regarding this manuscript.

References

Ackers, G. K. (1970), *Advan. Protein Chem. 24,* 343.
Andrews, P. (1970), *Methods Biochem. Anal. 18,* 1.
Brostrom, C. O., Corbin, J. D., King, C. A., and Krebs, E. G. (1971), *Proc. Nat. Acad. Sci. U. S. 68,* 24–44.
Brostrom, M. A., Reinmann, E. M., Walsh, D. A., and Krebs, E. G. (1970), *Advan. Enzyme Regul. 8,* 191.
Corbin, J. D., Brostrom, C. O., King, C. A., and Krebs, E. G. (1972), *J. Biol. Chem. 247,* 7790.
Davis, B. J. (1964), *Ann. N. Y. Acad. Sci. 121,* 404.
Erlichman, J., Rubin, C. S., and Rosen, O. M. (1973), *J. Biol. Chem. 248,* 7607.
Gill, G. N., and Garren, L. D. (1970), *Biochem. Biophys. Res. Commun. 39,* 335.
Glynn, I. M., and Chappell, J. B. (1964), *Biochem. J. 90,* 147.
Huang, L. C. (1974), *Biochim. Biophys. Acta 358,* 281.
Kumon, A., Yamamura, H., and Nishizuka, Y. (1970), *Biochem. Biophys. Res. Commun. 41,* 1290.
Langan, T. A. (1973), *Advan. Cyclic Nucl. Res. 3,* 94.
Laurent, T. C., and Killander, J. (1964), *J. Chromatogr. 14,* 317.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193,* 261.
Sawyer, W. H., and Puckridge, J. (1973), *J. Biol. Chem. 248,* 8429.
Tao, M., Salas, M. L., and Lipmann, F. (1970), *Proc. Nat. Acad. Sci. U. S. 67,* 408.
Trotta, P. P., Pinkus, L. M., Haschemeyer, R. H., and Meister, A. (1974), *J. Biol. Chem. 249,* 492.
Vesterberg, O. (1971), *Methods Enzymol. 22,* 389.
Walsh, D. A., and Ashby, C. D. (1973), *Rec. Progr. Horm. Res. 29,* 329.
Weber, K., and Osborn, M. (1969), *J. Biol. Chem. 244,* 4406.
Winstead, J. A., and Wold, F. (1965), *Biochemistry 4,* 2145.
Yamamura, H., Nishiyama, K., Shimomura, R., and Nishizuka, Y. (1973), *Biochemistry 12,* 856.

Thermodynamics of Complex Formation between Nicotinamide Adenine Dinucleotide and Pig Skeletal Muscle Lactate Dehydrogenase†

Hans-Jürgen Hinz* and Rainer Jaenicke

ABSTRACT: Formation of the binary complex between the reduced coenzyme nicotinamide adenine dinucleotide (NADH) and pig skeletal muscle lactate dehydrogenase (LDH, EC 1.1.1.27) has been investigated by calorimetric and equilibrium dialysis techniques in 0.2 M potassium phosphate buffer (pH 7.0) at various temperatures. Analysis of thermal titration curves at two temperatures (25 and 31.5°) shows that the experimental enthalpy data can be rationalized assuming four independent and equivalent binding sites for the tetrameric enzyme. Binary complex formation is characterized by a negative temperature coefficient, Δc_p, of the binding enthalpy, which amounts to -1300 ± 53 cal/(deg mol of LDH) in the temperature range of 5–31.5°. Despite the slightly smaller standard deviation resulting when polynomial regression analysis of the second degree is applied to the temperature dependence of the enthalpy values, binding enthalpies seem to be adequately represented in the temperature range studied by the equation $\Delta H = -1.3T + 2.3$, kcal/mol of LDH, T referring to the temperature in °C. By combination of the results obtained from equilibrium dialysis and calorimetric studies a set of apparent thermodynamic parameters for binding of NADH to LDH in 0.2 M potassium phosphate buffer at pH 7 has been established.

Binding of the coenzyme NADH to lactate dehydrogenase (LDH) is associated with conformational changes of the complex as demonstrated by X-ray determinations (McPherson, 1970; Adams *et al.*, 1973). This direct experimental evidence supports results from earlier kinetic investigations which showed the binding reaction of coenzyme and apoenzyme to be best interpretable by a mechanism involving a fast diffusion controlled bimolecular step followed by a slower monomolecular interconversion (Czerlinski and Schreck, 1964). Unless compensatory effects occur, one should expect the conformational changes to be reflected in

† From Biochemie II, Fachbereich Biologie, Universität Regensburg, D 8400 Regensburg, West Germany. *Received August 19, 1974.* This work was supported by grants of the Deutsche Forschungsgemeinschaft.