Li, S. L., & Yanofsky, C. (1973) *J. Biol. Chem. 248*, 1837–1843.
Lim, V. I. (1974) *J. Mol. Biol. 88*, 873–894.
Miles, E. W., & Moriguchi, M. (1977) *J. Biol. Chem. 252*, 6594–6599.
Miles, E. W., & Higgins, W. (1978) *J. Biol. Chem. 253*, 6266–6269.
Miles, E. W., Fairwell, T., & Higgins, W. (1979) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 38*, 563.
Pisano, J. J., Bronzert, T. J., & Brewer, H. B., Jr. (1972) *Anal. Biochem. 45*, 43–59.
Rice, R. H., & Means, G. E. (1971) *J. Biol. Chem. 246*, 831–832.
Shapiro, M., & Schultz, A. (1971) *Anal. Chem. 43*, 398–405.
Swank, R. T., & Munkres, K. D. (1971) *Anal. Biochem. 39*, 462–477.
Yanofsky, C., & Crawford, I. P. (1972) *Enzymes, 3rd Ed. 7*, 1–31.
Yutani, K., Ogasahara, K., Sugino, Y., & Matsushiro, A. (1977) *Nature (London) 267*, 274–275.
Zimmerman, C., Appella, E. A., & Pisano, J. J. (1977) *Anal. Biochem. 77*, 569–573.

Possibility of Shape Conformers of the Protein Inhibitor of the Cyclic Adenosine Monophosphate Dependent Protein Kinase†

John M. McPherson, Susan Whitehouse, and Donal A. Walsh*

ABSTRACT: The heat-stable, protein inhibitor of the cyclic adenosine monophosphate (cAMP) dependent protein kinase [Walsh, D. A., Ashby, C. D., Gonzalez, C., Calkins, D., Fischer, E., & Krebs, E. (1971a) *J. Biol. Chem. 246*, 1977–1985] has been purified to homogeneity from rabbit skeletal muscle by preparative electrophoresis. Employing a more sensitive assay system, we detected multiple charged forms of the inhibitor on diethylaminoethyl chromatography; the form that has been further characterizd is the predominant species in skeletal muscle comprising greater than 70% of the total. The apparent molecular weight of the protein inhibitor, as determined by Sephadex G-75 gel exclusion chromatography, is 22 000 in initial cellular extracts and at all stages during the purification prior to the final purification step of preparative gel electrophoresis, after which the homogeneous protein exhibits a molecular weight of 11 000. These two forms are designated I and I′, respectively. The I form migrates with an apparent molecular weight of 10 000 on nondenaturing gel electrophoresis and of 10 500–11 500 on sodium dodecyl sulfate ($NaDodSO_4$) gel electrophoresis; the I′ form migrates with an apparent molecular weight of 6500–8300 on $NaDodSO_4$ electrophoresis and has a minimum molecular weight of 10 400 by amino acid analysis. Taking into account the anomalous behavior displayed by low molecular weight proteins with the various techniques employed, we suggest that the I and I′ forms of the protein inhibitor may represent shape conformers.

A heat-stable, protein inhibitor of the cAMP-dependent protein kinase, which was partially purified from rabbit skeletal muscle, has been described by Walsh et al. (1971a). This inhibitor had an isoelectric point of 4.1, was precipitated but not inactivated by addition of trichloroacetic acid to 5%, was heat stable to 95 °C, and displayed an apparent molecular weight of ~26 000 on Sephadex G-75. The inhibitor blocked the activity of the cAMP-dependent protein kinase by direct interaction with the free catalytic subunit of the kinase; there was no displacement of the regulatory subunit from the holoenzyme, nor was there formation of a stable inhibitor–holoenzyme complex. These results demonstrated that the inhibitor interacts with the cAMP-dependent protein kinase only after cyclic nucleotide promoted dissociation (Ashby & Walsh, 1972, 1973). The interaction of the inhibitor and catalytic subunit has been shown to be readily reversible (Ashby & Walsh, 1973). Donnelly et al. (1973a,b) have attributed to the inhibitor protein a stimulatory activity toward the cGMP-dependent protein kinase. Subsequent studies by Kuo & Kuo (1976), however, have shown that the cAMP-dependent protein kinase inhibitor and the cGMP-dependent protein kinase modulator are distinct entities. The latter observation is consistent with the protein inhibitor interacting only with the catalytic subunit of the cAMP-dependent protein kinase following dissociation of the holoenzyme and the activation of the cGMP-dependent protein kinase not occurring by a dissociation mechanism (Gill et al., 1976; Lincoln et al., 1977). The modulator has recently been reported to act by interaction with the protein substrate (Shoji et al., 1978).

Since the time of the initial reports (Walsh et al., 1971a; Ashby & Walsh, 1972, 1973), several investigators have described the presence of heat-stable protein kinase inhibitors in various tissues that appear different from that first described. Demaille et al. (1977) purified a heat-stable protein inhibitor from rabbit skeletal muscle of molecular weight 11 000 based on Sephadex G-75 chromatography, $NaDodSO_4$ gel electrophoresis, and amino acid analysis. Weber & Rosen (1977) reported that the inhibitor from bovine cardiac muscle exhibited an apparent molecular weight of ~23 000. Szmigielski et al. (1977) and Costa (1977) have reported the presence of two types of inhibitors of protein kinases in various tissues of molecular weights 24 000 and 14 500. Beale et al. (1977) purified a heat-stable protein kinase inhibitor from rat testis of an apparent molecular weight in the range of 19 000–26 000. A primary purpose of the work described in this paper is to clarify the issue of the molecular size of the inhibitor protein. The heat-stable inhibitor initially described by Walsh et al. (1971a) has been purified to apparent homogeneity by means of preparative gel electrophoresis, and

† From the Department of Biological Chemistry, School of Medicine, University of California, Davis, Davis, California 95616. *Received June 22, 1979.* This work was supported by Grants AM 21019 and AM 13613 from the National Institutes of Health.

0006-2960/79/0418-4835$01.00/0

the physicochemical characteristics of this inhibitor are described and compared with those of the inhibitor preparations discussed above.

Experimental Procedures

Assay for the Protein Inhibitor of the cAMP-Dependent Protein Kinase. The protein inhibitor was assayed by a modification of the procedure described previously (Ashby & Walsh, 1972). Inhibitor fractions to be assayed were diluted in 10 mM Mes buffer, pH 6.8, containing 0.5 mg/mL bovine serum albumin. To 30 μL of inhibitor was added 20 μL of the catalytic subunit of the protein kinase (specific activity 3.0 μmol of PO_4 incorporated into histone IIA per mg of protein per min) diluted appropriately (vide infra) in 10 mM Mes buffer, pH 6.8, containing histone type IIA (30 mg/mL). To each assay tube was added 20 μL of 100 mM Mes buffer, pH 6.8, containing 20 mM magnesium chloride plus 15 mM β-mercaptoethanol. The assay was initiated by the addition of 10 μL of 1 mM [γ-^{32}P]ATP (80–100 dpm/pmol). Incubation was for 10 min at 30 °C. Phosphate incorporation into histone was determined by the filter paper (Whatman ET 31) method of Reimann et al. (1971) using a 50-μL aliquot of the reaction mixture. A reaction mixture containing all the assay components with the exception of the enzyme was utilized as a blank to determine the nonspecific [γ-^{32}P]ATP binding. The use of histone type IIA as the protein substrate instead of casein as described previously (Ashby & Walsh, 1972) provided greater reproducibility between assays of the protein inhibitor and also enhanced the sensitivity of the assay since significantly greater $^{32}PO_4$ incorporation occurs. The pH of the assay (pH 6.8) provides for both maximum activity of the protein kinase and maximum sensitivity to inhibition by the protein inhibitor (Figure 1A). As previously indicated (Ashby & Walsh, 1972), the absolute decrease in catalytic activity of the protein kinase is dependent upon not only the amount of inhibitor but also on the amount of protein kinase in the assay. In the protein inhibitor assay based on histone, the catalytic subunit was added to the incubation tubes to provide an uninhibited velocity of 49 ± 7 pmol of PO_4 incorporated per min. This value was based on the observation that the specific activity of the protein kinase catalytic subunit, using histone type IIA as the protein substrate, was ~7 times that obtained when casein was used as the protein substrate and that for the initial inhibitor assay established utilizing casein as substrate (Ashby & Walsh, 1972) protein kinase was present at an assay concentration that catalyzed the incorporation of 7 ± 1 pmol of PO_4 incorporated per min into casein. This has permitted direct comparison between the original and new assays for the protein inhibitor. For the histone-based assay one unit of inhibitor activity is defined as that amount which decreases the control velocity by 7 pmol of PO_4 incorporated per min. This unit of inhibitor activity is, within experimental error, identical with that of our previous publications (Ashby & Walsh, 1972, 1973). The characteristics of the inhibitor standard curve are as presented previously (Ashby & Walsh, 1972); an equivalent example with histone is indicated in Figure 7A (vide infra). For the rapid approximation of inhibitor concentration over a broader concentration range, such as for the assay of column chromatographic eluates, velocity vs. inhibitor curves were replotted on a log–logit scale according to Beale et al. (1977).

Although the protein inhibitor acts as a competitive inhibitor of the protein kinase, presumably by binding to some component of the protein substrate binding site (Demaille et al., 1977; and vide Figure 7B–D), the loss of protein kinase catalytic activity, as may occur by denaturation upon storage,

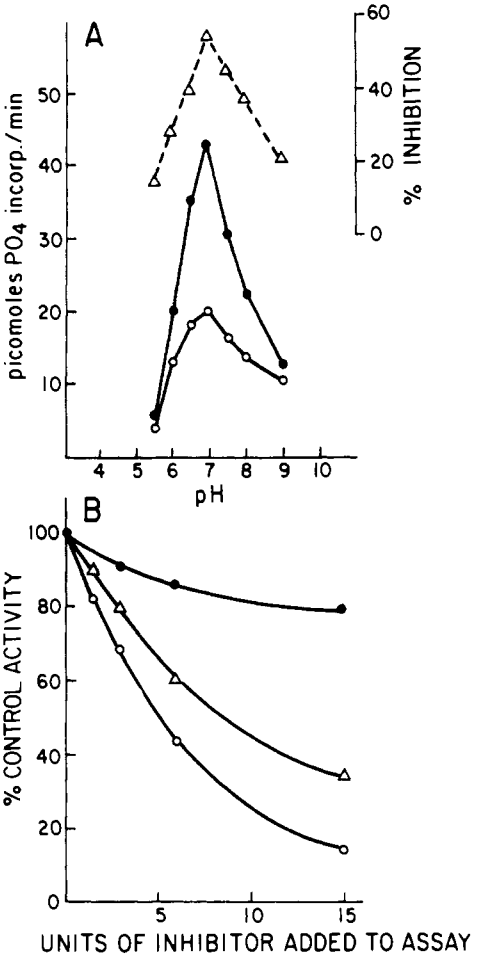

FIGURE 1: Characteristics of the protein inhibitor assay. (A) pH profile of inhibitory activity and the effect of inhibitor on protein kinase activity. The assays were performed as indicated under Experimental Procedures with the exception that the buffer of all components of the reaction mixture was replaced with Pipes at an equivalent concentration and at the indicated pH values. Aliquots of histone type IIA and bovine serum albumin were adjusted to the indicated pH and dialyzed against 10 mM Pipes at the corresponding pH values. The catalytic subunit was added to each incubation tube to provide an uninhibited velocity of 40 pmol of PO_4 incorporation/min at pH 6.8: (●) control values; (○) plus 5 units of protein inhibitor; and (△) percentage inhibition at the pH value tested. (B) Effect of protein inhibitor on catalytic subunit preparations of differing specific activities induced by storage at 4 °C over an 8-month period. The specific activities of the catalytic subunits utilized were (○) 3.2, (△) 1.2, and (●) 0.1 units/mg, respectively. The assays were performed by the standard procedure with sufficient protein kinase added to give a control incorporation of 49 ± 7 pmol/min. Activity is expressed as a percent of each control, which was 51.5, 53.5, and 44.5 pmol for △, ●, and ○, respectively.

has been observed to be not necessarily associated with an equivalent decrease in catalytic subunit–inhibitor interaction. A greater increment of protein inhibitor is required to effect an equivalent decrease in protein kinase activity for a partially denatured enzyme (Figure 1B). If unrecognized, this could compromise the accuracy of the inhibitor assay. All assays were therefore performed with the catalytic subunit of the protein kinase with a specific activity of no less than 2.5 units/mg.

To ensure that the observed inhibition of protein kinase activity, as detected in the various experimental probes, was not due to spurious components other than the protein inhibitor, we tested all samples for sensitivity to trypsin. Samples were incubated for 30 min at 30 °C with 0.15 mg/mL trypsin. The proteolytic reaction was terminated by addition of a

threefold excess of soybean trypsin inhibitor and the inhibitory activity redetermined.

Analytical Gel Electrophoresis. $NaDodSO_4$ gel electrophoresis was performed according to the method of Weber & Osborn (1969) using 12% polyacrylamide gel. Nondenaturing gel electrophoresis was performed according to the method of Ornstein (1964) and Davis (1964). Molecular weight determination in nondenaturing gels was done in accordance with the method of Hedrick & Smith (1968) in 7, 9, 11, and 13% nondenaturing slab gels using the buffer system described by Davis (1964). The position of the protein inhibitor in the gel was determined by elution and assay (vide infra). Slopes from plots of log (relative mobility) vs. percent gel concentration were determined by linear least-squares regression analysis using as standards insulin (6000), myoglobin (17 500), soybean trypsin inhibitor (22 000), carbonic anhydrase (31 000), ovalbumin (44 000), and BSA (68 000). Isoelectric focusing was performed by using a slight modification of the method of Catsimpoolas (1968). The system employed gels containing 7% acrylamide, 0.2% bis(acrylamide), and a final concentration of 4% ampholytes with a pH gradient of 3–5. By use of a voltage of 100 V, the inhibitor reached equilibrium within 12 h at room temperature. The pH gradient of the gels was determined by elution of 4-mm sections into 1 mL of H_2O. Protein bands were visualized on all types of gels by staining with 0.25% Coomassie blue R_{250}. It was necessary to soak isoelectric focusing gels in 40% methanol and 7% acetic acid for 12 h prior to staining in order to remove ampholytes.

Detection of Protein Inhibitor Subsequent to Gel Electrophoresis. The position of the inhibitor in nondenaturing gels and isoelectric focusing gels was determined by one of two procedures.

(*a*) *Method 1.* Immediately after electrophoresis, gels were sliced into 2-mm sections and placed in 3-mL disposable plastic tubes containing 200 μL of 0.2 M Mes buffer, pH 6.5. The tubes were sealed and shaken on an orbital shaker overnight at 4 °C. Aliquots (5–30 μL) from elution tubes were then withdrawn and assayed for inhibitory activity. Inhibitory activity was tested for trypsin sensitivity. Recovery of activity using this method ranged from 40 to 80%.

(*b*) *Method 2.* Trypsin-sensitive inhibitory activity was also detected for gels that had been stained in Coomassie blue R_{250} and subsequently destained. This was accomplished by slicing the destained gel into 2-mm sections, eluting the slices into 0.2 mL of 30% acetic acid, and shaking the elution tubes on an orbital shaker for 12 h at room temperature. At the end of this period of time, a 100-μL aliquot was withdrawn from each elution tube and individually lyophilized. Samples were then resuspended in 0.3 mL of deionized, distilled water and lyophilized. This lyophilized material was redissolved in 30 μL of 10 mM Mes buffer containing 0.5 mg/mL BSA (pH 6.8), and these fractions were assayed for inhibitory activity by the standard procedure. Recovery of activity using this method ranged from 10 to 20%.

The position of the inhibitor in $NaDodSO_4$ gels was determined either by method 2 or by the procedure described by Demaille et al. (1978). For the latter, which will be referred to as method 3, inhibitor was eluted from 2-mm slices into 200 μL of deionized, distilled water. Recovery of activity using this method ranged from 30 to 60%.

Protein Concentration. Generally, protein concentration was determined either according to the method of Lowry et al. (1951) or Bradford (1976). The protein concentration of the homogeneous inhibitor was based on amino acid analysis.

Materials. Histone type IIA, histone IIAS, bovine serum albumin fraction V, ATP, 2-(*N*-morpholino)ethanesulfonic acid (Mes), and piperazine-*N*,*N*′-bis(2-ethanetrypsinsulfonic acid) (Pipes) were purchased from Sigma. DE-52 anion-exchange resin and ET-31 filter paper were purchased from Whatman. Sephadex G-75 was obtained from Pharmacia. Materials for electrophoresis were purchased from Bio-Rad. Ampholytes for isoelectric focusing were purchased from Brinkmann. [γ-^{32}P]ATP was prepared according to the method of Walsh et al. (1971b). Myoglobin fragments used as standards for $NaDodSO_4$ gel electrophoresis were prepared by CNBr hydrolysis and were gifts of Dr. L. Waxman, University of California, Davis.

The catalytic subunit of cAMP-dependent protein kinase from bovine cardiac muscle was purified to homogeneity according to the method of Beavo et al. (1974). The specific activity of the purified protein was in the range of 3.0–3.5 μmol of phosphate incorporated into histone IIA per min per mg of protein. The enzyme was stored at −20 °C in 50% glycerol, 5 mM Mes buffer, pH 6.8, 50 mM sodium chloride, and 7 mM β-mercaptoethanol. cGMP-dependent protein kinase was purified to homogeneity from bovine lung according to the method described by Lincoln et al. (1977); the specific activity of the purified protein was 0.8×10^5 pmol of phosphate incorporated into histone IIA per min per mg of protein; the assay procedure used was that of Lincoln et al. (1977).

Results

Purification of the Protein Inhibitor of the cAMP-Dependent Protein Kinase to Homogeneity. The protein inhibitor has been purified to homogeneity by a modification and extension of the previously described procedure (Walsh et al., 1971a). This procedure has identified multiple species of the protein inhibitor by DEAE chromatography; the predominant species of skeletal muscle inhibitor is that which has been obtained in homogeneous form. The previously described preparation of purified protein inhibitor contained a mixture of the isoforms (Demaille et al., 1977).

Briefly, the preparation procedure used as adapted from Walsh et al. (1971a) is as follows. Six- to eight-pound female New Zealand White rabbits were sacrificed by cervical dislocation and exsanguinated. Skeletal muscle from hind limbs and back was removed and placed on ice. The muscle was ground in a chilled meat grinder and homogenized in 2.5 volumes (mL/g) of cold 4 mM EDTA (pH 7.0). The homogenate was centrifuged at 7000*g* for 30 min and the resulting supernatant filtered through glass wool. The pH of the solution was adjusted to 6.1 with 1 N acetic acid, and subsequently the solution was centrifuged at 7000*g* for 30 min. The supernatant was neutralized with 6 N NH_4OH, heated to 95 °C in a stainless steel bucket with overhead stirring, and then cooled to 10 °C in an ice–water bath. This solution, containing large amounts of denatured protein, was filtered through a Büchner funnel using S & S no. 588 filter paper. Trichloroacetic acid (Cl_3AcOH) (100%) was added rapidly to this clarified heat filtrate to a final concentration of 5%, and the mixture was stirred for 30 min at 4 °C. The precipitate, containing inhibitor, was collected by centrifugation at 7000*g*. This precipitate was resuspended in 50–100 mL of deionized, distilled water with a Teflon Potter–Elvejhem homogenizer. The suspension was neutralized with 6 N NH_4OH and dialyzed in Spectrapor 1 dialysis tubing against 10 L of 5 mM KPO_4 buffer, pH 7.0, containing 1 mM EDTA (3 changes), for a total of 18 h. The sample was then centrifuged at 34000*g* for 15 min. The supernatant was adjusted to pH 5.0 with 1 N acetic acid and stirred for 30 min. At the

Table I: Summary of Purification Data for the Protein Inhibitor

fraction	vol (mL)	total protein (mg)	sp act.[b] (units/mg of protein)	yield[b] (%)	x-fold purification
initial extract[a]	9000	1.6×10^5	6.2–11.6		1
heat filtrate	7600	3.3×10^3	$(3.0–4.2) \times 10^2$	100	43[c]
5% Cl_3AcOH precipitate	90	1.9×10^3	$(0.4–1.1) \times 10^3$	30–80	76
DE-52 eluate, peak I-2	2.2	4.9	$(3.5–6.7) \times 10^4$	8–22	6000
preparative electrophoresis	1.5	0.1	$(2.5–3.6) \times 10^5$	3–8	35000

[a] Preparation from 3.7 kg of rabbit skeletal muscle. [b] Ranges indicated for three typical preparations. [c] As indicated in the text, accuracy for determination of the activity in the initial extract is compromised by the presence of protein kinase substrates and trypsin-insensitive inhibitors; this value is based on the assumption of 100% yield at the initial step.

end of this time, the precipitate was removed by centrifugation at 34000*g* for 30 min. The supernatant was applied to a DE-52 column (1.5 × 30 cm) which had been equilibrated in 5 mM sodium acetate buffer, pH 5.0. The column was washed with 250 mL of this buffer and subsequently developed with a 1-L sodium acetate gradient (pH 5.0) between 0.005 and 0.35 M. The elution profile is indicated in Figure 2A. Although this procedure does not differ by substantial methodology from that described previously (Walsh et al., 1971a), the employment of a more sensitive and technically simplified assay [based on histone phosphorylation compared to a three-stage assay of phosphorylase activation; vide Experimental Procedures and Walsh et al. (1971a)] has permitted the detection of multiple peaks of heat-stable, trypsin-labile, inhibitory activity. On the basis of 10 preparations and utilization of the methods described above, three peaks have been consistently detected that elute at conductivities of 2.2, 2.7, and 3.2 mmhos; these have been designated as forms I-1, I-2, and I-3, respectively. On occasion, minor peaks eluting at conductivities other than those of the three designated forms have been detected. Although the relative amount of activity of the I-1, I-2, and I-3 forms has varied between preparations, the reproducible observation made is that form I-2 is the major species present in rabbit skeletal muscle, usually representing greater than 70% of the total activity detected from the column.

Purification of the I-2 inhibitor fraction to homogeneity was achieved by preparative gel electrophoresis. The fractions eluted from the DE-52 chromatography containing the I-2 inhibitor were pooled, neutralized to pH 7.0 by addition of 1 N NaOH, and dialyzed against 1 mM Mes buffer, pH 6.8, using Spectrapor 3 dialysis tubing. Samples were then lyophilized and redissolved in 1 to 2 mL of distilled water. Electrophoresis was performed with a Buchler preparative gel electrophoresis apparatus (Poly-Prep 200) by modification of the gel system as described by Ornstein (1964) and Davis (1964). The I-2 inhibitor was eluted as a single symmetrical peak (Figure 2B); the minor peak of activity that precedes the major fraction is the I-3 inhibitor fraction. Fractions containing inhibitory activity were concentrated by one of two methods. In the first method, inhibitor fractions were neutralized and exhaustively dialyzed in Spectrapor 3 dialysis tubing against 1 mM Mes buffer, pH 6.8. The protein was lyophilized and the resulting powder dissolved in 2 mL of deionized, distilled water. In the second method, inhibitor fractions were adjusted to pH 5.0 with 1 N acetic acid and applied to a 1.5-mL column of DE-52 cellulose equilibrated with 5 mM sodium acetate buffer, pH 5.0. The resin was washed with 50 mL of the buffer with which it had been equilibrated, and the inhibitor was subsequently eluted from the column in 3 mL by 0.3 M sodium acetate buffer, pH 5.0. The sample was briefly dialyzed against 10 mM Mes buffer, pH 6.8, using Spectrapor 3 dialysis tubing. This latter method, which also effectively removed glycine that was present as a component of the electrophoresis buffers, was used for the preparation of the protein inhibitor for amino acid determinations.

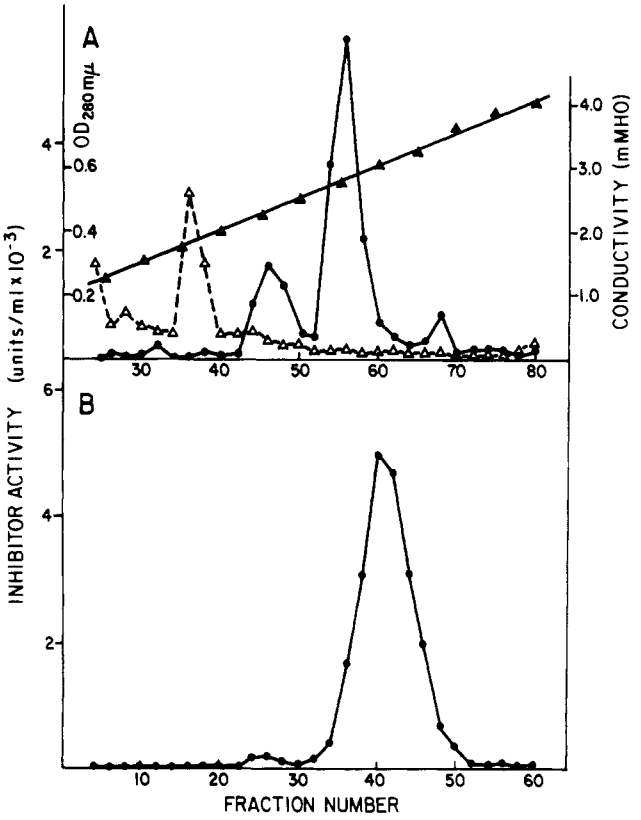

FIGURE 2: Purification profiles of the protein inhibitor. (A) A DE-52 cellulose column (1.5 × 30 cm) was equilibrated in 5 mM sodium acetate buffer, pH 5.0. Protein extracted from 3.7 kg of rabbit skeletal muscle, prepared as indicated under Results, was applied, and the column was washed with 250 mL of the equilibration buffer and developed with a linear gradient (1000 mL) of sodium acetate buffer, pH 5.0, between 5 mM and 0.35 M. Fractions (8 mL) were assayed for trypsin-sensitive inhibitory activity (●), for protein by absorbance at 280 nm (△), or for conductivity (▲). (B) Preparative gel electrophoresis was performed by using a Buchler preparative gel electrophoresis apparatus (Poly-Prep 200) with the gel system described by Ornstein (1964) and Davis (1964). The running gel was 128 mL in total volume of 8% acrylamide in 0.38 M Tris-HCl, pH 8.9. The stacking gel was 10 mL in total volume of 2.5% acrylamide in 0.063 M Tris-HCl, pH 6.7. The upper reservoir buffer was 0.004 M Tris and 0.038 M glycine, pH 8.3. The lower reservoir buffer and collecting buffer was 0.01 M Tris and 0.096 M glycine, pH 8.3. The upper and lower reservoir buffers were pumped at 1.5 mL/min, and the collecting buffer was pumped at 1 mL/min. The protein inhibitor sample, eluted from DE-52, was prepared as indicated under Results and diluted with an equal volume of sample buffer comprised of 50% glycerol and 0.25 M Tris-HCl, pH 6.7. 5–10 mg of protein was applied. The sample was stacked at 200 V for 90 min, and electrophoresis was performed at 350 V. The protein inhibitor eluted within 15 h; 3-mL fractions were collected following the elution of the dye front.

A summary of the purification procedure is provided in Table I; the procedure yielded 0.1–0.2 mg of inhibitor protein

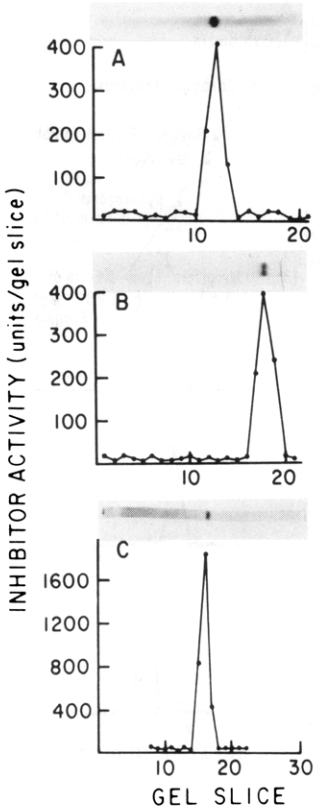

FIGURE 3: Polyacrylamide gel electrophoresis of the purified protein inhibitor of the cAMP-dependent protein kinase. (A) 12% $NaDodSO_4$ gel–20 μg of protein; (B) 7% nondenaturing gel–20 μg of protein; and (C) isoelectric focusing gel (pH 3–5)–15 μg of protein. The conditions of electrophoresis are as presented under Experimental Procedures. Inhibitor activity was detected by method 2 for (A) and (B) and by method 1 for (C).

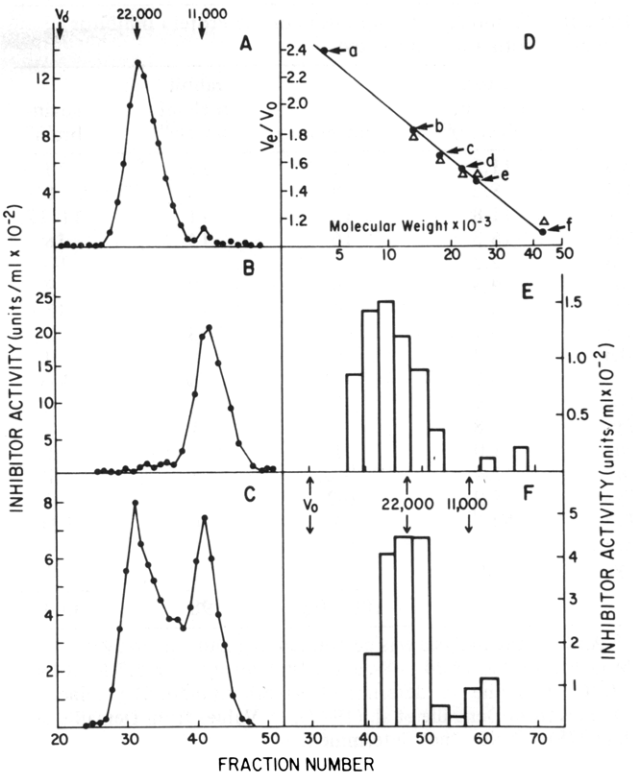

FIGURE 4: Sephadex G-75 column chromatography of the protein inhibitor. (A–C) Sephadex G-75 chromatography on a column (0.9 × 50 cm) equilibrated in 10 mM ammonium acetate, pH 7.0. (A) 4010 units of I-2 inhibitor eluted from DE-52 (Figure 2A); (B) 10 000 units of homogeneous protein inhibitor as eluted from preparative gel electrophoresis (Figure 2B); and (C) 2000 units of inhibitor I-2 eluted from DE-52 plus 2500 units of homogeneous protein inhibitor as eluted from preparative gel electrophoresis. The recoveries of inhibitory activity from the chromatographs were 75% (A), 50% (B), and 68% (C), respectively. The fraction volume was 0.6 mL. (E and F) Sephadex G-75 chromatography on a column (2.5 × 40 cm) equilibrated in 5 mM Mes buffer, pH 6.8, containing 20 mM sodium chloride. (E) 4 mL of initial tissue extract and (F) 4 mL of heat filtrate were applied to the column. The fraction volume was 2.4 mL. The fractions from (F) were pooled as indicated by the bar graphs, dialyzed for 24 h against 1 mM Mes buffer, pH 6.8, lyophilized, resuspended in H_2O, and assayed as described under Experimental Procedures. The fractions from (E) were pooled and heated to 95 °C for 5 min, and the denatured protein was removed by centrifugation prior to dialysis, lyophilization, and assay. For each, a volume of elution buffer equal to that of the separate pools was treated by an identical procedure to serve as a control for nonspecific buffer effects on the assay. (D) Molecular weight standard curve. Data points are for the 0.9 × 50 cm column (●) used for (A–C) and for the 2.5 × 40 cm column (△) used for (D) and (E), respectively. Standards were the following: (a) glucagon (3500); (b) cytochrome *c* (12 500); (c) myoglobin (17 500); (d) soybean trypsin inhibitor (22 000); (e) chymotrypsinogen (25 000); and (f) ovalbumin (44 000). The molecular weight standard curve was plotted according to the method of Whitaker (1963).

from 3.7 kg of rabbit skeletal muscle. The greatest variation in recovery of activity was observed with the Cl_3AcOH precipitation step; recovery was enhanced if the 100% Cl_3AcOH was added rapidly rather than by the dropwise addition of the acid solution to the heat filtrate. In those preparations where recovery was poor, a significant proportion of the trypsin-sensitive inhibitory activity could be detected as Cl_3AcOH-soluble activity even upon subsequent addition of Cl_3AcOH to the supernatant solution to a final concentration of 15% w/v. (Such an observation would be compatible with the conversion of the inhibitor from I to I′ form, vide infra.) The quantitative accuracy of the purification data (Table I) is compromised by the difficulty encountered in determining the amount of inhibitor in cell extracts that also contain cAMP-dependent and -independent protein kinases as well as endogenous substrates for each and by the presence of trypsin-insensitive inhibitor(s) in the heat filtrate. The purified inhibitor could be stored at −20 °C for at least 6 months without loss of activity.

The purified inhibitor protein preparation was homogeneous (Figure 3) by the criteria of nondenaturing gel electrophoresis on 7% acrylamide gels (also on 9, 11, and 13% gels; data not presented), by $NaDodSO_4$ gel electrophoresis, and by isoelectric focusing gel electrophoresis. With each of these techniques only a single band of Coomassie blue stained protein was detected; for each gel system there was coincident migration of the protein with trypsin-labile inhibitor activity (Figure 3). Nondenaturing gel electrophoresis would have detected the presence of inhibitor forms I-1 and I-3 in the I-2 preparation (S. Whitehouse, J. M. McPherson, and D. A. Walsh, unpublished experiments). The amino acid composition of the homogeneous inhibitor protein is presented in Table II. Since its composition differs little from those of the preparations of Demaille et al. (1977, 1978), which contain mixtures of isoforms, it would appear that the differences between the forms must reside in only minimal amino acid substitutions. The data of amino acid composition indicated that the protein inhibitor is composed of 100–102 amino acids, lacks cysteine and methionine, and has a minimum molecular weight of 10 400.

Apparent Molecular Size of the Protein Inhibitor. As determined by Sephadex G-75 gel exclusion chromatography, the apparent molecular weight for the I-2 form of the inhibitor purified up to and inclusive of DE-52 chromatography was 22 000 (Figure 4A). This value is within experimental error

Table II: Amino Acid Composition of the Protein Inhibitor Compared with Those Reported

	I′-2 form (residues/ molecule)[a]	integer	rabbit skeletal muscle[b]	bovine brain[c]
Asx	11.7	12	12	13
Thr	8.2	8	8	8
Ser	11.8	12	11	11-12
Glx	16.3	16	14	18
Pro	2.3	2	1	3-4
Gly	9.3	9	9	9
Ala	13.7	14	15	13
Val	3.1	3	3	3
Met	0.2	0	0	0
Ile	4.7	5	5	4-5
Leu	6.9	7	7	6-7
Tyr	0.8	1	1	1
Phe	1.0	1	1	1
His	1.4	1-2	1	1
Lys	4.9	1-2	4	5
Trp	ND[d]	5	0	ND
Arg	4.5	4-5	6	4
Cys	0.0	0	0	0
		100-102	98	100-104

[a] Amino acid analysis was performed in a Durrum analyzer (Model D-500) after 24-, 48-, and 72-h hydrolyses in 5.7 N HCl at 110 °C. Cysteine was determined as (carboxymethyl)cysteine. [b] Values from Demaille et al. (1977). [c] Values from Demaille et al. (1978). [d] ND = not determined.

of that reported previously from this laboratory for the protein purified to an equivalent stage (Walsh et al., 1971a). This value was also observed for the protein inhibitor at all prior stages of purification inclusive of that immediately extracted from the tissue (parts E and F of Figure 4). For this latter experiment, due to the low inhibitor concentrations in tissue extracts and subsequent dilution by gel filtration, the fractions were pooled and concentrated by lyophilization prior to assay; the fractions pooled are indicated by the bar graphs. To obtain sufficient material, we used a larger column than that for the analytical measurements of Figure 4A; the standarization of each is indicated (Figure 4D). As depicted (parts E and F of Figure 4), essentially all of the inhibitor in initial extracts of muscle was in the 22 000 molecular weight form, although a small amount of a lower molecular size component may have been present.

The final purification procedure of preparative gel electrophoresis resulted in the conversion of the inhibitor to a form of apparent molecular size of 11 000 daltons by gel exclusion chromatography (Figure 4B). [With some preparations a low percentage of the 22 000-dalton species was also present.[1]] Gel filtration of a mixture of the two forms effected their separation (Figure 4C). Thus, the observation that the apparent size of the inhibitor was changed during the purification process could not be ascribed to the removal of spurious nonspecific components which resulted in the modification of the elution profile. On the basis of standard Stokes radii for ovalbumin (27.3 Å), chymotrypsinogen (22.4 Å), myoglobin (20.7 Å), and cytochrome *c* (16.4 Å) (Andrews, 1970), the Stokes radii for the 22 000 and 11 000 apparent molecular weight forms of the protein inhibitor are 21 and 15 Å, respectively. These two forms of the protein inhibitor are denoted as I and I′, respectively.[2]

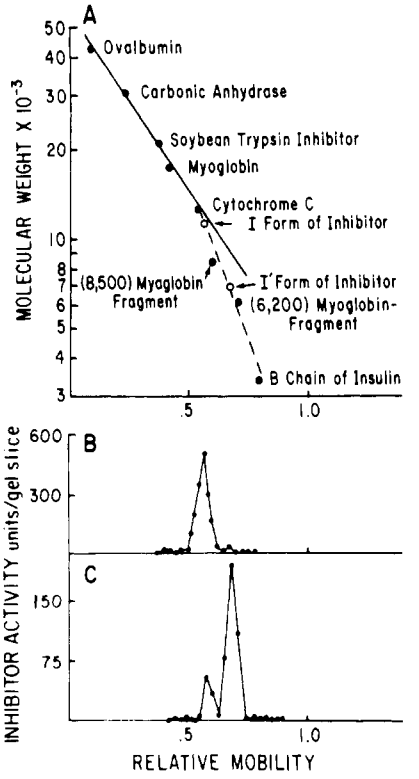

FIGURE 5: Molecular weight determination of I and I′ forms of the protein inhibitor based on $NaDodSO_4$ gel electrophoresis. (A) Standard curve and (B and C) mobility of the I and I′ forms detected by elution and assay. (B) 2000 units of protein inhibitor purified through the DE-52 column chromatography step and (C) 800 units of protein inhibitor as eluted from preparative gel electrophoresis were applied, respectively. Inhibitor was detected by using method 3 (Experimental Procedures).

The apparent molecular weights of the I and I′ forms of the protein inhibitor have been determined by both nondenautring and denaturing gel electrophoresis procedures. By the method of Hedrick & Smith (1968), inhibitor fraction I, as detected by elution from the gel and subsequent activity assay, exhibited a molecular weight of 10 000. It is to be noted, however, that although the inhibitor protein applied to the electrophoresis gel was that of a preparation purified up to and inclusive of the DE-52 chromatogaphy step and, in consequence, was in the I form as indicated by gel filtration (Figure 4A), there is no assurance that it was not converted to the I′ form by the procedure of acrylamide electrophoresis. The components to which the protein would be exposed in analytical gel electrophoresis are identical with those of preparative gel electrophoresis (Figure 2B), and the latter promoted the conversion of the I to the I′ form (cf. parts A and B of Figure 4). The molecular weight of the homogeneous I′ form was likewise examined by the procedure of Hedrick & Smith (1968) (data not shown); no difference in the size of the I and I′ forms could be detected by this procedure.

The molecular size of the I and I′ forms of the inhibitor protein was also examined by the method of Weber & Osborne (1969) on $NaDodSO_4$ gel electrophoresis. For proteins of molecular weight less than 12 000, the accuracy of the determination is compromised by the deviation from linearity of the standard curve (Figure 5A). This deviation is caused,

[1] Although the average yield of inhibitor protein was low and in some preparations it would not be possible to distinguish the conversion of I to I′ from the selective loss of I, usually the total amount of I′ present subsequent to preparative electrophoresis was several-fold greater than that applied.

[2] Each of the charge isomers of the protein inhibitor, I-1, I-2, and I-3, exhibits the molecular characteristics of the size conformers I and I′ (S. Whitehouse, J. M. McPherson, and D. A. Walsh, unpublished experiments). The nomenclature used for these six forms is I-1, I′-1, I-2, I′-2, I-3, and I′-3.

at least in part, by the different geometrical configuration of large and small protein–$NaDodSO_4$ complexes. As the axial ratio of the protein–$NaDodSO_4$ complex for small proteins approaches a value of 1.0, the typical rodlike particle of the higher molecular weight protein–$NaDodSO_4$ complexes approximates closer to that of a sphere as the length of the rod equals the diameter (Reynolds & Tanford, 1970; Fish et al., 1970). The I and I′ forms of the inhibitor protein exhibited apparent molecular weights on $NaDodSO_4$ electrophoresis in the ranges of 10 500–11 500 and 6500–8300, respectively; the high and low values for each range were calculated from the extrapolated linear portion of the standard curve and the actual curve with low molecular weight standards, respectively. Although the molecular weights of the I and I′ forms cannot be calculated with accuracy from $NaDodSO_4$ gel electrophoresis, the mobility of each was different and the two forms clearly separated (parts B and C of Figure 5), thus providing evidence that the I and I′ forms are different in size and/or shape.

Demaille et al. (1977) have previously reported that protein inhibitor purified to homogeneity by affinity chromatography with a Sepharose–cAMP-dependent protein kinase catalytic subunit column had a molecular weight by either gel filtration or $NaDodSO_4$ gel electrophoresis of 11 000. Furthermore, in attempting to delineate the apparent discrepancy between their observations and those previously presented by this laboratory (Walsh et al., 1971a), Demaille et al. (1977) reported that protein inhibitor extracted by nondenaturing conditions and purified only by DE-52 chromatography also exhibited a molecular weight of 11 000. We have further investigated these apparent differences. Protein inhibitor was partially purified by the procedure of Demaille et al. (1977) up to and inclusive of elution from DE-52. The fractions containing inhibitor were pooled, neutralized, dialyzed extensively against 10 mM ammonium acetate (pH 7.0), and lyophilized, and the protein was subsequently resuspended in 10 mM ammonium acetate, pH 7.0. The latter steps are identical with those utilized by Demaille et al. (1977) for the partial purification of the inhibitor as used to study the size of the protein in crude tissue extracts. As indicated (Figure 6A), protein prepared by such a procedure exhibited a molecular size of 11 000 by gel exclusion chromatography. The procedure by which this latter inhibitor protein was prepared only differs substantially from that used in the current studies in the dialysis against ammonium acetate and subsequent lyophilization [as used by Demaille et al. (1977)] and Cl_3AcOH precipitation (as used in the current reported studies, Table I). As already documented (cf. parts A, E, and F of Figure 4), treatment with Cl_3AcOH does not affect the molecular size of the inhibitor protein. The question thus remained as to why the inhibitor migrated in the I′ form using the procedure described by Demaille et al. (1977). Although the question has not been fully answered, the results shown in parts B and C of Figure 6 demonstrate that dialysis of the protein inhibitor against 10 mM ammonium acetate followed by lyophilization of the sample can at least partially convert I to the I′ form. Lyophilization in the presence of ammonium bicarbonate mediates a similar conversion, although a similar treatment with ammonium chloride or sodium acetate had no such effect. This conversion was not observed with inhibitor preparations containing high concentrations of contaminating proteins. The I form is stable to gel filtration in 10 mM ammonium acetate (Figure 4A).

Kinetic Characterization of the Mode of Inhibition of the cAMP-Dependent Protein Kinase by the I and I′ Forms of the

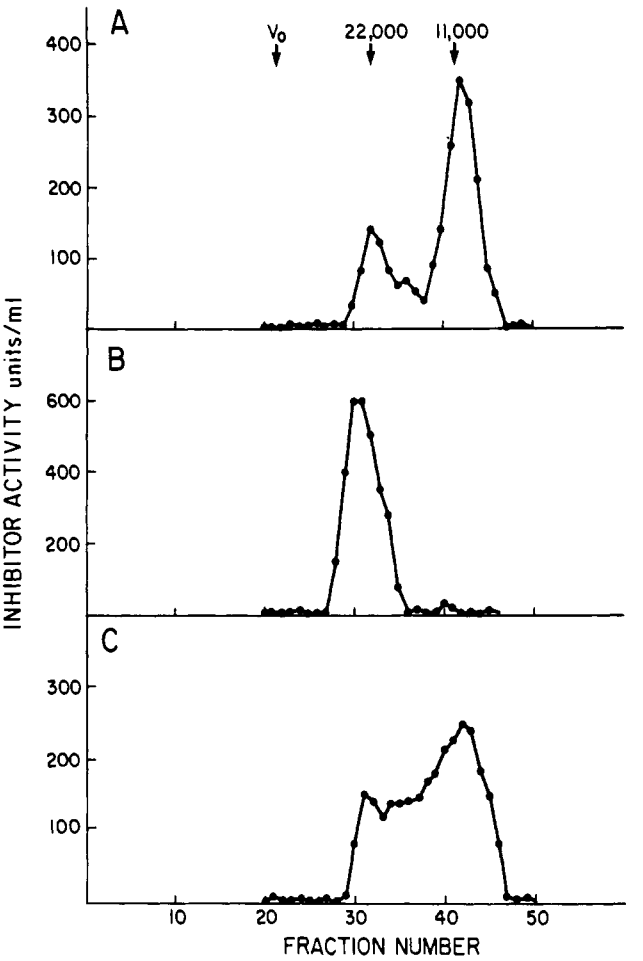

FIGURE 6: Effect of purification conditions and treatment with ammonium acetate on the molecular size of the protein inhibitor. Sephadex G-75 gel exclusion chromatography was performed as indicated for the experiment of Figure 4A–C. (A) Protein inhibitor was purified by the procedure of Demaille et al. (1977) up to and inclusive of elution from DE-52. The fractions eluted from DE-52 containing inhibitor were pooled, neutralized, dialyzed extensively against 10 mM ammonium acetate (pH 7.0), and lyophilized, and the protein was suspended in 10 mM ammonium acetate, pH 7.0, and applied to the Sephadex G-75 column. (B) Control protein inhibitor (1600 units), purified by the standard procedure through the DE-52 chromatography step (vide Table I). (C) Protein inhibitor (2500 units) purified as for (B) was dialyzed against 10 mM ammonium acetate, lyophilized, and resuspended in 10 mM ammonium acetate before being applied to the column. Arrows indicate the elution positions of proteins of molecular weight 22 000 and 11 000, respectively, based on standard curve data (vide Figure 4D).

Protein Inhibitor. The initial characterization of the mechanism of inhibition of protein kinase by the protein inhibitor, based on Lineweaver–Burk plots, had depicted the kinetic mechanism as noncompetitive with respect to both substrates (Walsh et al., 1971a). Subsequently, as has been previously pointed out by Demaille et al. (1977), Henderson (1972) has shown that for inhibitors with high affinity constants such a method of analysis is invalid. With a preparation that was presumably of the I′ form, Demaille et al. (1977) have shown by the Henderson procedure that the inhibition is competitive with respect to protein substrate with a K_i equal to 2.1 nM. From testing by the graphical method devised by Dixon (1972), it is apparent (Figure 7A) that, under typical conditions for investigating the interaction of the inhibitor protein (presented for the I form) with the protein kinase, a significant fraction of the inhibitor is enzyme bound; as classified by Segel (1975), the inhibitor–protein kinase interaction is appropriately evaluated as a "tightly bound

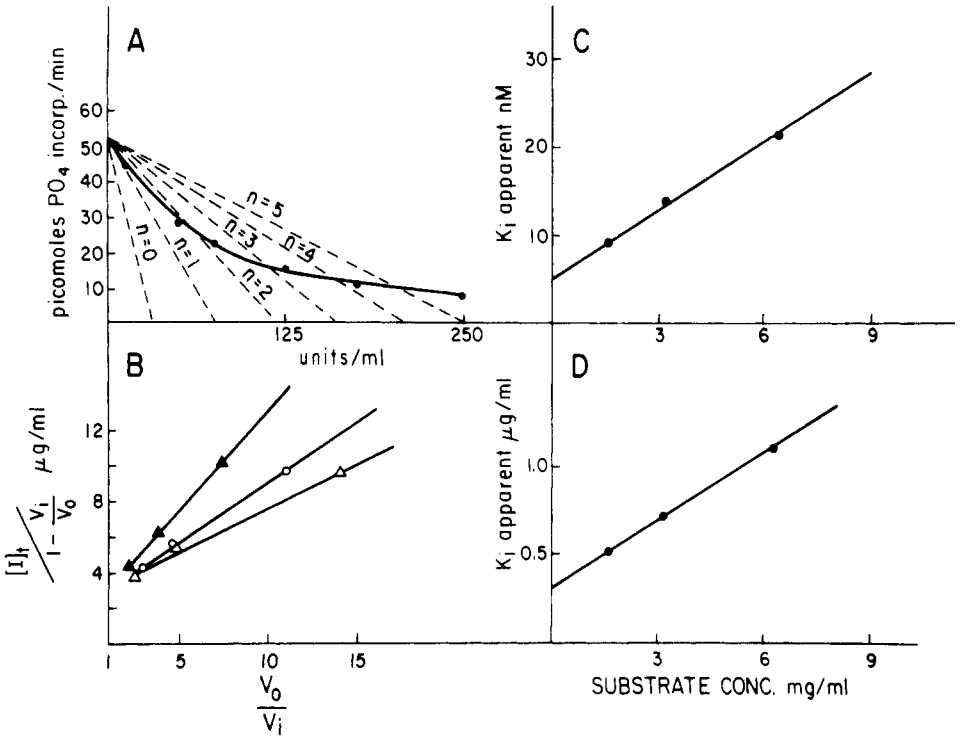

FIGURE 7: Kinetic characteristics of the interaction of the I and I′ forms of the protein inhibitor with the cAMP-dependent protein kinase. (A) Plot of the protein kinase reaction velocity vs. the inhibitor concentration as delineated by Dixon (1972). Assay conditions were as for the standard assay with the exception that the histone substrate concentration was 2 mg/mL to give $v_0 = 0.9V_{max}$. Dashed lines for values of $n = 2$–5 are as provided for by Dixon to give increments of apparent $K_i = 43.75$ inhibitor units/mL. Dashed lines for $n = 0$ and 1 are calculated from equivalent increments. (B) Henderson plot for I form of inhibitor (DE-52 eluate) at histone concentrations of 1.6 (△), 3.2 (○), and 6.4 (▲) mg/mL. (D) Replot of slope for the data of (B). (C) Replot of slopes of Henderson plots for purified I′.

inhibitor". The Henderson plot (Henderson, 1972) for the I–protein kinase interaction is presented in Figure 7B, and replots of the slope of the Henderson plot vs. protein substrate concentration are presented for the I′ and I forms in parts C and D of Figure 7, respectively. In confirmation of the results of Demaille et al. (1977), I′ is a competitive inhibitor; the calculated K_i (5 nM) is within experimental error of that reported. From the Henderson plot, the I form is likewise shown to be a competitive inhibitor of the protein kinase with respect to the protein substrate. This is demonstrated by the fact that the apparent K_i, given by the slope of the lines (Figure 7B), becomes greater with increases in substrate concentration (Figure 7D). Since the I form has yet to be obtained homogeneous (vide supra), it has only been possible to estimate the K_i for the I–protein kinase interaction based on the assumption of the degree of purity of the fraction (17%, Table I). With this assumption, the K_i for the I form is estimated to be 5 nM.[3] This kinetic characterization thus suggests that the I and I′ forms are equally effective inhibitors of the protein kinase, each acting by a mechanism that is competitive with the protein substrate and having approximately equal affinity for the protein kinase.

Szmigielski et al. (1977) have described the presence of an inhibitor of molecular weight 15 000 that blocks the action of both cAMP-dependent and cGMP-dependent protein kinases. The effect of both the I and I′ forms on lung cGMP-dependent kinase has been tested. Inhibitor, present in the assay of cGMP-dependent protein kinase at inhibitor/kinase molar ratios of 1.75 or 7.0 for I′ or 7.0 and 19.0 for I, modified the activity from a control value of 18.6 pmol incorporated/min to values of 20.0, 21.0, 21.5, and 23.0 pmol incorporated/min, respectively. Hence, no inhibition of activity was detected, and possibly a slight activation of the enzyme occurred.

Discussion

The data of this paper serve to delineate the apparent different forms of the protein inhibitor of the cAMP-dependent protein kinase that have been described by this and other laboratories (Walsh et al., 1971a; Demaille et al., 1977; Szmigielski et al., 1977; Costa, 1977; Beale et al., 1977; Weber & Rosen, 1977). We report the presence of three charge isomers (I-1, I-2, and I-3) in extracts from rabbit skeletal muscle (Figure 2A). The apparent molecular weight of the protein inhibitor at the point of earliest detection, i.e., the crude extract from muscle (parts E and F of Figure 4) and for partially purified I-2 (Figure 4A), is 22 000 by gel exclusion chromatography. This is identical with the molecular size of the protein inhibitor described previously by this laboratory (Walsh et al., 1971a). This current paper also reports that the I-2 form of the inhibitor can be converted into a species (designated I′-2) that exhibits an apparent molecular weight of 11 000 by gel exclusion chromatography (Figures 4B and 6C). This interconversion does not represent formation of either I-1 or I-3 from I-2 since each of the former exhibits a molecular weight of 22 000 by gel exclusion chromatography (S. Whitehouse, J. M. McPherson, and D. A. Walsh, unpublished experiments); in addition, the I-1 species has also been demonstrated to be convertible to a form of molecular weight 11 000 (i.e., I′-1). To date, only the I′ form has been obtained as a homogeneous protein, due to the conversion of I into I′ by the final purification step of preparative gel electrophoresis. Other efforts to purify I without its concomitant conversion to I′ have been unsuccessful.

[3] Calculation of the K_i for the I form assumes either a true molecular weight of 11 000 or a dimeric two active site species of 22 000. If I is a monomer of M_r 22 000 or a dimer of 22 000 with one active site, the calculated K_i value would be 10 nM.

Demaille et al. (1977) reported the purification of the protein inhibitor from skeletal muscle by affinity chromatography using a Sepharose–protein kinase catalytic subunit column. The characteristics of this protein of molecular size by both gel exclusion chromatography and $NaDodSO_4$ gel electrophoresis, amino acid composition, and K_i identify this as the I′ form of the protein inhibitor (cf. Figures 4 and 7 and Table II). Poly(acrylamide) gel electrophoretic profiles presented by Demaille et al. (1977) indicated a splitting of the peak into two fractions which probably represent I′-1 and I′-2. Demaille et al. (1977) also reported that the protein inhibitor was present in crude tissue extracts as the I′ (M_r 11 000) species. In all probability this was an erroneous conclusion since the method used by those workers to concentrate the protein inhibitor from crude cellular extracts (i.e., dialysis against ammonium acetate and subsequent lyophilization) has now been shown to convert I to the I′ form (parts B and C of Figure 6). Our data (parts E and F of Figure 4) indicate the I form to be the probable physiological species. The step in the Demaille et al. (1977) purification procedure that converts I to I′ in the preparation of homogeneous protein has not been determined; potentially it is the use of guanidine hydrochloride to elute the protein inhibitor from the catalytic subunit affinity column. It may also reflect the mechanism of interaction of the inhibitor with the catalytic subunit of the protein kinase.

Recent reports by Szmigielski et al. (1977) and Costa (1977) indicated the presence of two heat-stable inhibitors of the cAMP-dependent protein kinase in several different types of tissue. These inhibitors were distinguished by elution from Sephadex G-100. The type I inhibitor has an apparent molecular weight of ~24 000 based on molecular sieve chromatography and is presumed to correspond to the I form of the inhibitor. The type II inhibitor displayed an apparent molecular weight of 14 000–15 000 based on the same method and also differs from type I in that it was not precipitated by 5% Cl_3AcOH (Szmigielski et al., 1977; Costa, 1977). In addition, type II inhibited not only cAMP-dependent protein kinase but also cGMP-dependent protein kinase as well as certain other cyclic nucleotide independent protein kinases. The type II inhibitor of Szmigielski et al. (1977) and Costa (1977) is similar in molecular size to the I′ form reported here, but apparently the I′ form does not inhibit cGMP-dependent protein kinase. It is, however, worthwhile to note that whereas the I form is completely precipitated by 5% Cl_3AcOH, a significant amount (10–30%) of trypsin-sensitive inhibitory activity remains in the supernatant when the I′ form is treated by such a procedure (data not presented).

Weber & Rosen (1977) described the purification of the protein inhibitor from bovine myocardium. By gel filtration the molecular weight of this protein was 22 900, which corresponds to that of the I form. The preparation contained one major protein which exhibited a molecular weight of 22 200 by $NaDodSO_4$ gel electrophoresis, but because the inhibitor had been purified only 320-fold whereas in skeletal muscle, which contains essentially the same level of protein inhibitor activity as cardiac muscle (Ashby & Walsh, 1972), a 35 000-fold purification is required, it is doubtful that the protein identified by Weber & Rosen (1977) following $NaDodSO_4$ electrophoresis was the inhibitor.

Beale et al. (1977) have purified a heat-stable inhibitor of cAMP-dependent protein kinase from rat testis. This protein has a molecular weight of between 19 000 and 26 000 based upon the three criteria of gel filtration, $NaDodSO_4$ gel electrophoresis, and amino acid analysis. These properties and the marked differences in amino acid composition clearly distinguish the protein inhibitor of Beale et al. (1977) from either the I-2 or I′-2 forms reported in this current study. Other chromatographically separable forms of heat-stable inhibitory activity from rat testis were also detected by Beale et al. (1977), and they may correspond to those reported here for skeletal muscle.

Table III: Physical Parameters of the I and I′ Forms of the Protein Inhibitor

	Stokes radius[a] (Å)	f/f_0[b]	axial ratio[c]
I form	21	1.3	4
I′ form	15	1.0	1

[a] Determined by gel filtration. [b] Calculated assuming a molecular weight of 11 000 for each form and from the partial specific volume of 0.72 calculated from amino acid composition (Cohn & Edsall, 1965). [c] Derived from f/f_0.

This report has illustrated that the protein inhibitor can exist as forms I and I′. Only I′ has so far been obtained in a homogeneous form. I′ exhibits an approximate apparent molecular weight of 11 000 by gel filtration (Figure 4B), by the Hedrick & Smith (1968) technique with nondenaturing gel electrophoresis, and by amino acid analysis (Table II). The apparent molecular weight of I′ by $NaDodSO_4$ electrophoresis (Figure 5) appeared somewhat lower than 11 000, but this value may be compromised by the nonideal behavior of small proteins and/or an abnormal level of $NaDodSO_4$ binding to the protein inhibitor due to its amino acid composition. I′ would appear to be a globular protein of 15-Å Stokes radius (Table III).

Potential explanations for the different physicochemical characteristics of I′ and I include the following: (a) I is a dimer of I′; (b) native inhibitor is a molecule of M_r 22 000 whose primary structure has been cleaved during purification, and the nicked molecule (i.e., I) remains intact due to tertiary structural forces under nondisruptive conditions but may be disrupted to yield an active fragment (i.e., I′); (c) native inhibitor is a molecule of M_r 22 000 (I) that contains a covalent bond that is highly susceptible to cleavage to yield an active fragment (I′);[4] and (d) I and I′ have identical compositions and molecular weights but exist in different shape conformations. Of these alternatives, the first three are not readily compatible (vide infra) with the data presented whereas the last alternative is compatible with the data but, to the knowledge of these investigators, is not supported by past precedent with other proteins.[5]

A monomer–dimer relationship between I′ and I could have reasonably explained the observed different elution profiles of these two forms from Sephadex G-75 (Figures 4 and 6), and such a rationale would be consistent with the widespread observation that many proteins exist in multimeric forms. Such would also have provided a comparison with the dimeric structure of the regulatory subunit of the protein kinase, a protein that shares in common with the protein inhibitor the

[4] Alternatives b and c differ essentially only on the basis of whether cleavage of a key bond has occurred at the final stage of the purification or during the early stage of purification with the cleaved components of the molecule remaining bound. Such a situation as the latter is well documented for ribonuclease (Kalman et al., 1955).

[5] At least two other proteins which coelute with the protein inhibitor as the 22 000 molecular weight species following Sephadex G-75 chromatography (at an equivalent stage to that of Figure 4A) also exhibit, as detected by protein staining, a molecular weight of 11 000 on both $NaDodSO_4$ and nondenaturing gel electrophoresis.

ability to interact with and inhibit the catalytic subunit of this latter enzyme. A molecular weight of 11 000 for I by the Hedrick & Smith technique would be inconsistent with I being a dimer of 22 000, but since preparative gel electrophoresis resulted in the conversion of I to I′ (parts A and B of Figure 4), it would not have been unreasonable to assume that analytical nondenaturing gel electrophoresis would also have effected such a conversion. That the molecular weight of both I and I′ was ~8000–11 000 by $NaDodSO_4$ gel electrophoresis would also be compatible with a monomer–dimer relationship, but the clear separation of the two forms by this technique (Figure 5) would appear to preclude this model.

Similar rationale also argues against I′ being a fragment derived from I during extraction and/or purification. If I were a prenicked molecule held together by tertiary structure, the active inhibitory fragment identified by $NaDodSO_4$ gel electrophoresis should be identical for I (the prenicked molecule held together by tertiary structure) or for I′ (the active fragment isolated by preparative gel electrophoresis). In contrast, I and I′ are separated by this procedure (Figure 5). An alternate explanation would be that the active fragment I′ was cleaved from I either at the last stage of purification (preparative gel electrophoresis, Figure 2B) during nondenaturing analytical electrophoresis or by the treatment with ammonium acetate followed by lyophilization (parts B and C of Figure 6). Why a bond would be labile under such diverse conditions is difficult to rationalize. $NaDodSO_4$ gel electrophoresis data also argue against such an explanation. Since I and I′ migrate differently, I must be presumed to be intact; however, a reason must then be sought to explain why I exhibits a molecular weight of 10 000 on $NaDodSO_4$ electrophoresis but a molecular weight of 22 000 on gel filtration. To investigate further the possibility that I′ was a fragment either bound by tertiary structural forces and/or cleaved from I, we treated the I form with dimethyl suberimidate under the cross-linking conditions described by Davies & Stark (1970); such, however, did not conserve the molecular parameters that would have been consistent with a 22 000-dalton species.

The possibility compatible with all current information is that I and I′ are molecules of essentially identical composition but, because of some feature of primary, secondary, or tertiary structure, of different shape. I′ is proposed to be a molecule of 11 000 molecular weight that is globular with a Stokes radius of 15 Å. This is consistent with gel filtration elution, migration under the conditions of Hedrick & Smith (1968), and amino acid composition. I is proposed to be a molecule of 11 000 molecular weight, with a Stokes radius of 21 Å (Table II) due to its being an elongated molecule with an axial ratio of 4. Such data are compatible (Table III) with its behavior on gel filtration in comparison to standard globular proteins as a protein of apparent molecular weight 22 000; moreover, many proteins have been reported to have axial ratios of 4 or greater. The patterns of $NaDodSO_4$ gel electrophoresis of I′ and I (Figure 5) would be compatible with such a proposal if it were assumed that the forces that restricted I′ as a globular protein were not disrupted by $NaDodSO_4$ so that I′ was restricted from forming the typical rodlike $NaDodSO_4$–protein complex (Reynolds & Tanford, 1970). Treatment of I, as indicated above, with dimethyl suberimidate produced a complex $NaDodSO_4$ gel electrophoretic profile suggestive of the presence of varying degrees of restriction ranging from that of the I structure to that of the I′ structure (data not presented). If I and I′ are of the same molecular weight but of different molecular shapes, it would have to be assumed that such shape differences did not affect the inhibitory function of the molecule since the K_i values for I and I′ are identical (Figure 7). In that it has been proposed (Demaille et al., 1977) that the inhibitory function resides only in a small peptide fragment, such a feature would not be difficult to rationalize. The proposal that I and I′ may be two conformers of the same moiety is only one of several possible explanations of the data obtained to date. Elucidation of the correct explanation awaits the purification of I to homogeneity.

References

Andrews, P. (1970) *Methods Biochem. Anal. 18*, 1–53.

Ashby, C. D., & Walsh, D. A. (1972) *J. Biol. Chem. 247*, 6637–6642.

Ashby, C. D., & Walsh, D. A. (1973) *J. Biol. Chem. 248*, 1255–1261.

Beale, E. G., Dedman, J. R., & Means, A. R. (1977) *J. Biol. Chem. 252*, 6322–6327.

Beavo, J., Bechtel, P., & Krebs, E. G. (1974) *Methods Enzymol 38*, 299–308.

Bradford, M. (1976) *Anal. Biochem. 72*, 248–254.

Catsimpoolas, N. (1968) *Anal. Biochem. 26*, 480–482.

Cohn, E. J., & Edsall, J. T. (1965) in *Proteins, Amino Acids and Peptides*, pp 428–431, Hafner Publishing Co., New York.

Costa, M. (1977) *Biochem. Biophys. Res. Commun. 78*, 1311–1318.

Davies, G., & Stark, G. (1970) *Proc. Natl. Acad. Sci. U.S.A. 66*, 651–656.

Davis, B. (1964) *Ann. N.Y. Acad. Sci. 121*, 404–427.

Demaille, J. G., Peters, K. A., & Fischer, E. H. (1977) *Biochemistry 16*, 3080–3086.

Demaille, J. G., Peters, K. A., Strandjord, T. P., & Fischer, E. H. (1978) *FEBS Lett. 86*, 113–116.

Dixon, M. (1972) *Biochem. J. 129*, 197.

Donnelly, T. E., Kuo, J. F., Miyamoto, E., & Greengard, P. (1973a) *J. Biol. Chem. 248*, 199–203.

Donnelly, T. E., Kuo, J. F., Reyes, P L., Liu, Y., & Greengard, P. (1973b) *J. Biol. Chem. 248*, 190–198.

Fish, W. W., Reynolds, J., & Tanford, C. (1970) *J. Biol. Chem. 245*, 5166–5168.

Gill, G. N., Holdy, K. E., Walton, G. M., & Kaustein, C. B. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 3918–3922.

Hedrick, J. L., & Smith, A. J. (1968) *Arch. Biochem. Biophys. 126*, 155–164.

Henderson, P. J. (1972) *Biochem. J. 127*, 321.

Kalman, S. M., Linderstrøm-Lang, K., Ottesen, M., & Richards, F. M. (1955) *Biochim. Biophys. Acta 16*, 297–304.

Kuo, W., & Kuo, J. F. (1976) *J. Biol. Chem. 251*, 4283–4286.

Lincoln, T. M., Dills, W. L., & Corbin, J. D. (1977) *J. Biol. Chem. 252*, 4269–4275.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

Ornstein, L. (1964) *Ann. N.Y. Acad. Sci. 121*, 321–349.

Reimann, E. M., Walsh, D. A., & Krebs, E. G. (1971) *J. Biol. Chem. 246*, 1986–1995.

Reynolds, J., & Tanford, C. (1970) *J. Biol. Chem. 245*, 5161–5165.

Segel, I. (1975) in *Enzyme Kinetics*, pp 150–159, Wiley, New York.

Shoji, M., Brackett, N., Shapira, R., & Kuo, J. (1978) *J. Biol. Chem. 253*, 3427–3434.

Szmigielski, A., Guidotti, A., & Costa, E. (1977) *J. Biol. Chem. 252*, 3848–3853.

Walsh, D. A., Ashby, C. D., Gonzalez, C., Calkins, D., Fischer,

E., & Krebs, E. (1971a) *J. Biol. Chem. 246*, 1977–1985.
Walsh, D. A., Perkins, J. P., Bromstrom, C. O., Ho, E. S., & Krebs, E. G. (1971b) *J. Biol. Chem. 246*, 1968–1976.
Weber, K., & Osborn, M. (1969) *J. Biol. Chem. 244*, 4406–4412.
Weber, H., & Rosen, O. M. (1977) *J. Cyclic Nucleotide Res. 3*, 415–424.
Whitaker, J. R. (1963) *Anal. Chem. 35*, 1950–1953.

Inhibition of Pig Liver Methylenetetrahydrofolate Reductase by Dihydrofolate: Some Mechanistic and Regulatory Implications†

Rowena G. Matthews* and Bobbie J. Haywood

ABSTRACT: Methylenetetrahydrofolate reductase has been purified 3400-fold from pig liver. The enzyme exhibits ping-pong bi–bi kinetics during catalysis of NADPH–methylenetetrahydrofolate oxidoreduction. Dihydrofolate is an inhibitor of this reaction and is competitive with respect to methylenetetrahydrofolate. The K_i for dihydrofolate is 6 μM. The inhibition of NADPH–methylenetetrahydrofolate reductase activity is greatest when dihydrofolate is present, and similar concentrations of tetrahydrofolate, methyltetrahydrofolate, folic acid, methenyltetrahydrofolate, or 5-methyl-5,6-dihydrofolate are 2.5–10-fold less effective. These observations suggest that the enzyme-catalyzed reduction of methylenetetrahydrofolate may proceed by tautomerization to form an N^5-methyldihydrofolate derivative. However, the formation of N^5-methyl-7,8-dihydrofolate is precluded by our failure to see loss of tritium from C-6 of the pteridine ring of methylenetetrahydrofolate during reduction. Proton magnetic resonance spectroscopy studies on the methyltetrahydrofolate formed during enzymatic reduction of methylenetetrahydrofolate in deuterium oxide demonstrate that two atoms of deuterium are incorporated into the methyl group. We suggest that inhibition of NADPH–methylenetetrahydrofolate oxidoreductase activity by dihydrofolate may be important in the regulation of the flux of methylenetetrahydrofolate in vivo. Both thymidylate synthetase and methylenetetrahydrofolate reductase are found in the cytoplasm, as is dihydrofolate reductase. When thymidylate biosynthesis is occurring, the resulting increase in steady-state levels of dihydropteroylglutamate derivatives may serve to inhibit methylenetetrahydrofolate reductase, thus sparing methylenetetrahydropteroylglutamates for purine and pyrimidine biosynthesis.

Methylenetetrahydrofolate reductase (EC 1.1.99.15) catalyzes the reaction

$$\text{NADPH} + \text{H}^+ + \text{methylene–H}_4\text{PtGlu}_1 \rightarrow \text{NADP}^+ + \text{methyl–H}_4\text{PtGlu}_1 \quad (1)$$

This reaction, which is effectively irreversible in vivo (Katzen & Buchanan, 1965), commits one carbon units to the pathways of *S*-adenosylmethionine-dependent methylation in mammalian systems. The mammalian enzyme was first identified by Donaldson & Keresztesy (1962), who showed that FAD was required for maximal activity. It was purified about 900-fold by Kutzbach & Stokstad (1971). They demonstrated that the enzyme was inhibited by *S*-adenosylmethionine, a typical example of feedback inhibition by the final common product of a reaction pathway.

Methylenetetrahydrofolate reductase is one of five mammalian enzymes which are known to use methylenetetrahydrofolate as substrate. This substrate is essential for the biosynthesis of dTMP, catalyzed by thymidylate synthetase, for purine biosynthesis, which requires the conversion of methylenetetrahydrofolate to methenyl- and N^{10}-formyltetrahydrofolate, and for *S*-adenosylmethionine-dependent methylation reactions. Thus, the potential exists for competition between these pathways for methylenetetrahydrofolate.

The present studies were initiated to look for possible regulatory mechanisms which might exist to assure an equitable distribution of substrate between methylenetetrahydrofolate reductase and other competing pathways.

Experimental Procedure

Purification of Methylenetetrahydrofolate Reductase. The enzyme used in these experiments has been purified from pig liver by a new method. One kilogram of frozen pig liver was thawed and homogenized in a Waring blender in 2 L of 50 mM phosphate, pH 5.9, 0.3 mM in EDTA. The homogenate was adjusted to pH 5.9 and centrifuged at 20000*g* for 30 minutes. The pH of the supernatant was adjusted to pH 7.2, and 71 g of DEAE-52 (previously equilibrated with 50 mM phosphate buffer, pH 7.2, and sucked dry by aspiration on a Büchner funnel) was added. The suspension was stirred for 30 min, filtered on a Büchner funnel, and washed with 1 L of 50 mM phosphate buffer. The DEAE cake was then extracted twice with 500-mL volumes of 0.3 M phosphate buffer, pH 7.2, and the eluates were collected by filtration, combined, and applied directly to a 1.5 × 18 cm column of Affi-Gel Blue (Bio-Rad Laboratories). The column was rinsed with 100 mL of 0.3 M phosphate buffer, pH 7.2, and then eluted with a linear gradient of 0–10 mM NADPH. Fractions with maximal methylenetetrahydrofolate reductase activity were pooled, and the enzyme was collected by precipitation with 80% ammonium sulfate.

Assays of Methylenetetrahydrofolate Reductase. NADPH–menadione oxidoreductase activity was measured

† From the Veterans Administration Hospital and the Department of Biological Chemistry, The University of Michigan, Ann Arbor, Michigan 48109. *Received April 25, 1979.* This work has been supported by the Medical Research Service of the Veterans Administration and in part by Research Grant GM 24908 from the National Institute of General Medical Sciences, National Institutes of Health, U.S. Public Health Service, and by Michigan Memorial Phoenix Project 534. A preliminary account of some of this work was presented at the Sixth International Symposium on the Chemistry and Biology of Pteridines, September 1978, La Jolla, CA.

* This work was performed during the tenure of an Established Investigatorship of the American Heart Association and with funds contributed in part by the Michigan Heart Association. Address correspondence to this author at the Biophysics Research Division, Institute of Science and Technology, The University of Michigan.

0006-2960/79/0418-4845$01.00/0