J. Enzyme Inhibition, 1993, Vol. 7, pp. 151–157
Reprints available directly from the publisher
Photocopying permitted by license only

A METHOD FOR COUNTING ACTIVE SITES OF CYCLIC AMP-DEPENDENT PROTEIN KINASE

G. DAVID McCLURE, JR., RAHEEL QAMAR and PAUL F. COOK*

Departments of Biochemistry and Molecular Biology and of Microbiology and Immunology, Texas College of Osteopathic Medicine, Fort Worth, Texas 76107, U.S.A.

(Received 21 May 1992)

A method has been developed for counting active sites of cyclic-AMP-dependent protein kinase. Known concentrations of a synthetic peptide similar to a fragment of the endogenous inhibitor of the kinase were included in otherwise routine assay mixes containing several different volumes of enzyme stock solution. The concentration of active sites of the catalytic subunit of the cyclic AMP-dependent protein kinase in the stock solution was then determined by fitting observed velocities to an equation that accounts for the presence of a tight-binding inhibitor. The method yielded estimates of catalytic subunit concentration comparable with those derived from more traditional measures of catalytic subunit concentration. Both purified and heterogeneous samples were assayed, since active-sites counting assumes only a mutually specific, high-affinity interaction between enzyme and inhibitor and does not require that samples be pure. In principle, the method can be adapted to other protein kinases for which a specific, tight-binding, reversible inhibitor is available.

KEY WORDS: Enzyme kinetics, tight-binding inhibitor, cyclic AMP-dependent protein kinase.

INTRODUCTION

The catalytic subunit (*C*)‡ of the heterotetrameric enzyme, adenosine 3′:5′-monophosphate-dependent protein kinase (EC 2.7.1.37), catalyzes transfer of the gamma-phosphoryl group from MgATP to the hydroxyl oxygen of appropriately placed serine or threonine residues in proteins and peptides. Abundant structural and mechanistic data have been gathered on *C*, so that it has become the most thoroughly characterized member of the protein kinase family.[2,3] In spite of this wealth of information, there has been no reliable way to determine the concentration of *C* in solutions of unknown composition. Below, is presented a method for counting *C* active sites that takes advantage of the availability of a reversible, tight-binding inhibitor[4] of *C* and makes use of Williams and Morrison's general treatment of enzyme-catalyzed reaction rates in the presence of such inhibitors.[5] Two main

* Correspondence.

† A preliminary report of this work was presented at the 75th annual meeting of the Federation of American Societies for Experimental Biology.[1]

‡ Abbreviations: *C*, the catalytic subunit of adenosine 3′:5′-monophosphate-dependent protein kinase; MOPS, 3-(*N*-morpholino)propanesulfonic acid; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

assumptions underlie the application of this method: (a) the affinity of the inhibitor for *C* far exceeds the affinity of the inhibitor for any other component of the solution; (b) the affinity of *C* for the inhibitor far exceeds the affinity of *C* for any other component of the solution. Where these assumptions are satisfied, the active-sites counting method can be used to determine the concentration of *C* in solution.

MATERIALS AND METHODS

DE-52 resin was purchased from Whatman. All other resins were bought from Bio-Rad. NADH was purchased from Boehringer Mannheim. All other buffers, reagents, and coupling enzymes were bought from Sigma.

The *C*-terminal amide form of the inhibitor heptadecapeptide[4] with sequence TYADWIASGRTGRRNAI-NH_2 was purchased from Multiple Peptide Systems (San Diego, California). The substrate heptapeptide with sequence LRRASLG was synthesized by solid-phase methods. Each peptide eluted as a single symmetrical peak when subjected to reverse-phase HPLC.

C was isolated from fresh bovine heart according to the method of Reimann and Beham[6] and further subjected to the affinity purification scheme of Olsen and Uhler.[7] Purity was assessed by SDS-PAGE: overloaded lanes on gels stained with Coomassie Brilliant Blue were inspected for minor bands. Affinity purification eliminated any components still present after the initial isolation that had given rise to minor bands.

The concentration of peptide substrate in stock solution was determined enzymatically according to the method of Cook *et al.*[8] The concentration of inhibitor peptide in stock solution was determined by measuring the absorbance at 280 nm and dividing the measured absorbance by the molar absorption coefficient of 6800 M^{-1} cm^{-1}. This value is the sum of the molar absorption coefficients for the *N*-acetyl methyl ester of tryptophan and for tyrosine in neutral aqueous solution.[9] The concentration of ATP in stock solution at pH 7 was determined by measuring the absorbance at 259 nm and dividing the measured absorbance by the molar absorption coefficient[10] of 15400 M^{-1} cm^{-1}.

The concentration of *C* in stock solution was estimated by two independent methods. The absorbance at 280 nm was measured, and the concentration of *C* was determined by dividing the measured absorbance by the molar absorption coefficient of 57000 M^{-1} cm^{-1} reported by Peters *et al.*[11] Also, the activity of *C* was measured by the coupled assay described by Cook *et al.*[8] in the absence of inhibitor. The measured activity was divided by the volume of the solution and by the benchmark figure for specific activity of purified *C*, 19 units per mg at 10 mM free Mg^{2+}, previously reported from this laboratory.[8] A value[12] of 40862 was used for the relative molecular mass of *C*.

Measurements of the initial velocity of the phosphoryl transfer from MgATP to peptide substrate catalyzed by *C* were made by performing the coupled assay described by Cook *et al.*[8] in quartz cuvettes of 1 cm path-length that held a final volume of 0.4 ml. Through the pyruvate kinase and lactate dehydrogenase reactions, the enzymatic couple links the production of MgADP by *C* to the oxidation of NADH. The absorbance at 340 nm was monitored continuously on a strip-chart recorder, offset to 0.2 full-scale, showing the output from a Gilford 260 spectrophotometer.

The standard concentrations and conditions chosen for the active-sites counting procedure were: 2 mM ATP, 0.3 mM peptide substrate (10 K_M), 12 mM $MgCl_2$,

0.25 mM NADH, 1 mM phospho(enol)pyruvate, 40 units lactate dehydrogenase, 20 units pyruvate kinase, 100 mM KCl, 100 mM MOPS, pH 7.0, 25°C. The concentration of the inhibitor peptide was varied as follows: 0, 6.2, 12.4, or 24.8 nM. All components of the assay mix other than the peptide substrate were incubated together for 5 min. After the background rate of ATP hydrolysis had been recorded during this 5-min period, the peptide substrate was added.

Initial velocity data were analyzed as described Cleland[13] and fitted using the equation

$$\log(v) = \log(V_{max}(AE - I - K + ((K + AE - I)^2 + 4KI)^{0.5})/2) \quad (1)$$

where v is the observed velocity, V_{max} is the maximum velocity, AE is the concentration of *C* active sites in the cuvette, I is the concentration of inhibitor in the cuvette, and K is the apparent inhibition constant for I under the conditions employed.

Below, estimates of parameters generated by the active-sites counting method are reported as mean ± standard error. Each of the other analytical procedures routinely employed in this laboratory has been shown to have a coefficient of variation $\leqslant 10\%$.

RESULTS

Linear initial rates were observed at all concentrations of inhibitor peptide employed in the active-sites counting procedure. The best fit of the data to equation (1) gave estimates of 1.34 ± 0.16 μM for the concentration of active sites in the enzyme stock solution and of 1.31 ± 0.08 nM for the K_i of the inhibitor peptide. A plot of observed velocity versus volume of enzyme stock solution added can be seen in Figure 1.

The active-sites counting procedure was used to estimate the concentration of *C* in a fraction of the cyclic-AMP eluate from a DE-52 column used for the isolation of *C* from a crude homogenate of bovine heart. This eluate, collected during the isolation procedure of Reimann and Beham,[6] consistently contains several polypeptide species revealed upon analysis by SDS-PAGE (data not shown). A volume of 21.3 μl of eluate gave a velocity of 3.4 μM/min when inhibitor was absent, and a velocity of 2.5 μM/min in the presence of 6.2 nM inhibitor. Estimates of the final concentration of *C* were made by locating the point on the appropriate inhibitor concentration curve in Figure 2 that had a y-value corresponding to the observed velocity. The x-coordinate of this point is the estimated final concentration of *C*. In each case, the final concentration of *C* was estimated to be between 5.4 and 5.6 nM when read off the graph, indicating a concentration of *C* in the DE-52 eluate of approximately 100 nM.

DISCUSSION

The traditional measures, absorbance and specific activity, gave estimates of 1.23 μM and 1.13 μM, respectively, for the concentration of *C* in the stock solution (data not shown). These estimates are comparable with the estimate of 1.34 μM for active sites in stock solution derived from the active-sites counting procedure presented here. The closeness of the estimates derived from the traditional and novel procedures is consistent with the presence of one active site per molecule of *C*. Taylor and

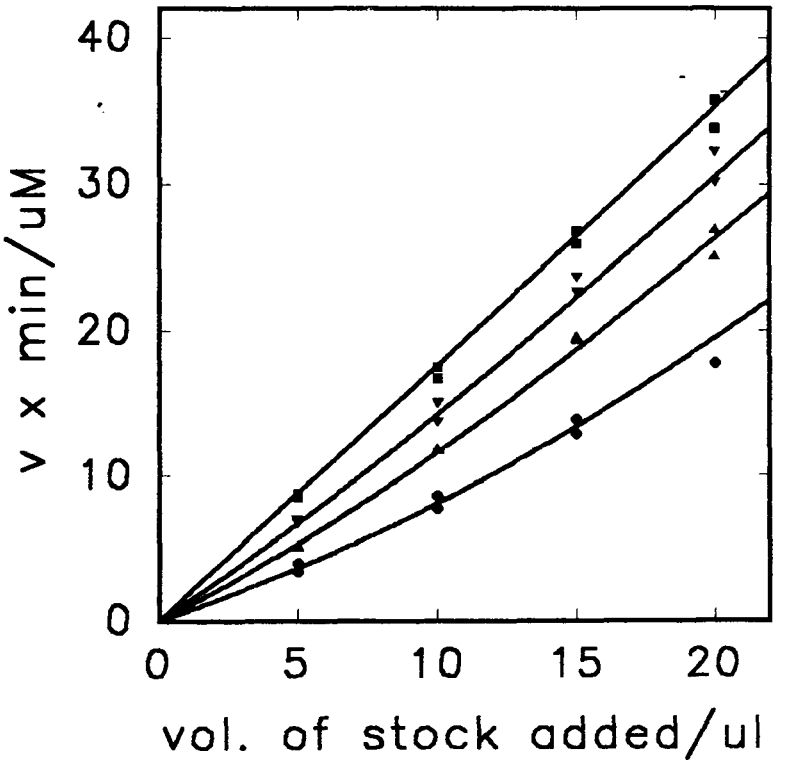

Figure 1 Velocity as a function of amount of enzyme added at various levels of inhibitor. Velocity is plotted as a function of the volume of stock enzyme solution added to give a final volume of 0.4 ml in which the concentration of inhibitor is 0 nM (■), 6.2 nM (▼), 12.4 nM (▲) or 24.8 nM (●). Experimental conditions are described in "Materials and Methods."

co-workers[3] have recently presented a crystal structure in which there is indeed one active site per molecule of *C*. The consistency of all these results indicates that the active-sites counting procedure yields accurate estimates of the concentration of *C*.

The active-sites counting procedure also generated an estimate of K_i for the inhibitor peptide of 1.3 nM which is in general agreement with the value of 1.0 nM reported by Glass *et al.*[4] The closeness of these two estimates provides further evidence in favor of the reliability of the procedure described.

The active-sites counting procedure was employed to estimate that the eluate from a DE-52 column contained *C* at a concentration of 100 nM. Moreover, the data in Figure 2 show that the fraction assayed contained no activity, other than *C*, that phosphorylated the peptide substrate. The presence of interfering activities not inhibited by the inhibitor peptide would have caused estimates of enzyme concentration to become progressively larger as inhibitor concentration was increased. This is because the added inhibitor would inhibit only *C* and would not stop phosphorylation by other kinases that might phosphorylate the substrate. Whenever the estimate of *C* concentration at non-zero inhibitor concentration is no greater

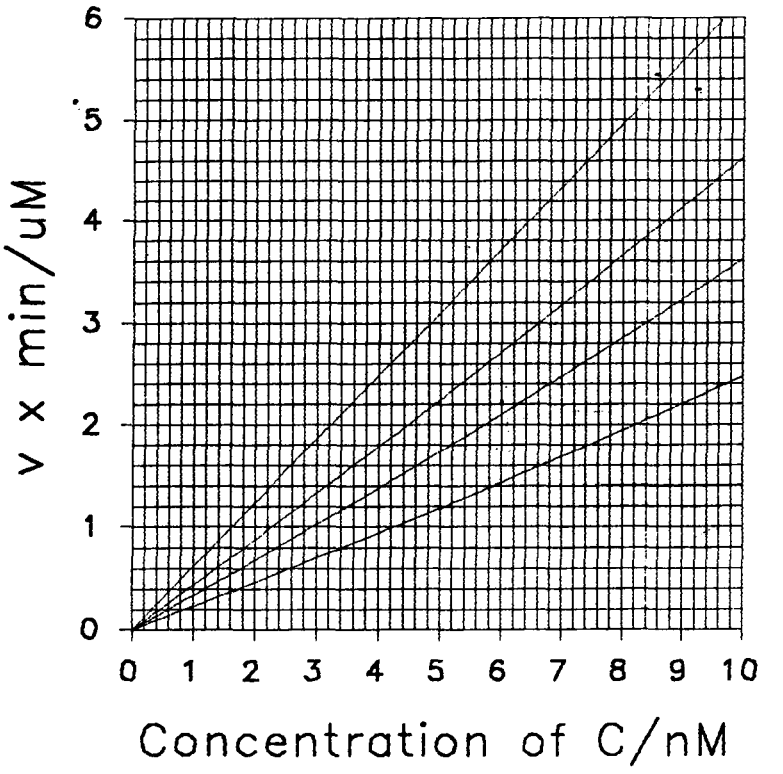

Figure 2 Nomogram showing relationship between velocity and enzyme concentration at various levels of inhibitor. Velocity is plotted as a function of enzyme concentration in the presence of 0 nM (top curve), 6.2 nM (next curve below), 12.4 nM (second curve from bottom) or 24.8 nM (botton curve) inhibitor. Each curve maps velocities on the ordinate to enzyme concentrations on the abscissa. A grid has been superimposed to facilitate the reading of values off the graph; e.g., the top curve, showing velocity when inhibitor is absent, maps an observed velocity of 3.4 μM/min on the ordinate to a point between 5.4 and 5.6 nM *C* on the abscissa. Likewise, the next curve below, showing velocity at 6.2 nM inhibitor, maps an observed velocity of 2.5 μM/min on the ordinate to a point between 5.4 and 5.6 nM *C* on the abscissa. Other experimental conditions are described in the text.

than the estimate generated in the absence of inhibitor, it can be concluded that the activity phosphorylating the peptide substrate is due to *C* alone.

The foregoing analysis requires two assumptions: (a) the affinity of the inhibitor for *C* far exceeds the affinity of the inhibitor for any other component of the solution; (b) the affinity of *C* for the inhibitor far exceeds the affinity of *C* for any other component of the solution.

The amino-acid sequence of the inhibitor peptide used in the active sites counting method differs from that of a well-characterized inhibitory fragment of the endogenous heat-stable inhibitor protein at only one residue.[4,14] The inhibitor peptide containing tryptophan at the P-11 position (see reference 15 for explanation of nomenclature) was chosen because of the felicitous UV spectral properties of tryptophan residues and because it had been reported as having a lower K_i than the parent peptide.[4] It

is otherwise identical in sequence to positions P−15 to P+1 of the well-studied fragment. The structure of the entire fragment, P−16 to P+3, bound to *C* has recently been presented.[15] High-affinity binding of the N-terminal portion of the fragment to *C* seems to depend on interactions between the phenylalanine residue at P−11 and a hydrophobic pocket formed by residues 235 to 239 on *C*. Scott *et al.*[16] have shown that neither the endogenous inhibitor protein nor another of its fragments (P−10 to P+9) inhibits cyclic GMP-dependent protein kinase, protein kinase C, myosin light-chain kinase, phosphorylase kinase, or casein kinase II. Taken as a whole, these data suggest a unique and specific interaction between the inhibitor peptide and *C*. It is therefore reasonable to assume that the inhibitor peptide will not bind anything so tightly as it binds *C*: that is, assumption (a) is in general satisfied. It is also reasonable to assume that, among endogenous ligands, only the regulatory subunits, when free of cyclic AMP, and the inhibitor protein will bind *C* as tightly as does the synthetic inhibitor peptide. Where these other proteins are absent, assumption (b) is in general satisfied.

The ability to detect active sites at nM levels makes active-sites counting appropriate for estimations of *C* in unfractionated eukaryotic cell extracts and tissue homogenates, where *C* may be present at concentrations of hundreds of nM.[17] Ashby and Walsh[18] have shown that dissociation of the holoenzyme tetramer is necessary for interaction of the endogenous inhibitor protein with *C*. By analogy, when the present method is applied to unfractionated samples from eukaryotic tissues, one should impose or induce an increase in the concentration of cyclic AMP to ensure holoenzyme dissociation and hence interaction between the synthetic inhibitor peptide and *C*.

Where present, the endogenous inhibitor protein will bind a portion of all the *C* in solution and render the bound *C* molecules unavailable for interaction with the synthetic inhibitor. Walsh and Ashby[19] have estimated that the inhibitor protein is bound to as much as 21% of the *C* present in some tissues. These authors arrived at this estimate by titration of *C* with inhibitor protein and extrapolation of the linear portion of the titration curve.[19] Whether their method or another is chosen, the binding of *C* by the inhibitor protein must always be accounted for in order not to underestimate the *C* concentration in tissues presumed to have high inhibitor protein concentrations whenever active-sites counting or any other assay based primarily on measurement of *C* activity is performed.

Active-sites counting is of more general utility than traditional methods of determining the amount of *C* present in solution. Unlike measurement of absorbance, active-sites counting does not require highly pure enzyme for accurate estimates of enzyme concentration. Unlike use of benchmark figures for specific activity, active-sites counting does not assume that the peptide substrate is being phosphorylated only by the cAMP-dependent protein kinase.

For similar reasons, active-sites counting can be adapted to utilize novel peptide substrates which may be subject to phosphorylation by a variety of protein kinases. The active-sites counting procedure will estimate the concentration of *C* in solution even if other kinases are present which also phosphorylate the substrate. This is due to the specificity of the interaction of the synthetic inhibitor peptide with *C* and not with other kinases.

If another kinase besides *C* is present which can phosphorylate the peptide substrate, an estimate of the relative proportions of *C* and the interfering activity can be obtained. Recall that, in the case of the DE-52 eluate, a velocity of 3.4 μM/min was observed in the absence of inhibitor and a velocity of 2.5 μM/min was observed at 6.2 nM

inhibitor (Figure 2). If half of the activity had been due to other kinases phosphorylating the peptide substrate, a velocity of 2.9 μM/min would have been observed in the presence of 6.2 nM inhibitor. If a velocity of 3.2 μM/min had been observed at 6.2 nM inhibitor, this would mean that 80% of the observed activity were due to other kinase activities. If only 20% of the activity were due to kinases other than *C*, a velocity of 2.7 μM/min would have been observed at 6.2 nM inhibitor. One could assay also at the higher inhibitor concentrations in order to confirm these estimates.

Recombinant *C* has been expressed in organisms (such as *S. cerevisiae*)[20] and cell lines (such as fibroblasts and anterior pituitary cells)[21] likely to contain several protein kinases of unknown substrate specificity. Active-sites counting allows estimation of the concentration of *C* even in a milieu of such uncertain composition.

In principle, the overall scheme of active-sites counting presented above can be adapted to estimate concentrations of catalytic sites of any protein kinase so long as a specific, tight-binding, reversible inhibitor is available. As such inhibitors are developed, active-sites counting procedures may be used to estimate the concentration of each of the protein kinases in various pathways of signal transduction.

Acknowledgements

This work was supported by grants from the National Institutes of Health (GM37057) and the Robert A. Welch Foundation (B1031) to P.F.C.

References

1. Qamar, R., McClure, G.D., Jr. and Cook, P.F. (1991) *FASEB Journal*, **5**, A799.
2. Taylor, S.S., Buechler, J.A. and Yonemoto, W. (1990) *Ann. Rev. Biochem.*, **59**, 971–1005.
3. Knighton, D.R., Zheng, J., Ten Eyck, L.F., Ashford, V.A., Xuong, N.-h., Taylor, S.S. and Sowadski, J.M. (1991) *Science*, **253**, 407–414.
4. Glass, D.B., Lundquist, L.J., Katz, B.M. and Walsh, D.A. (1989) *J. Biol. Chem.*, **264**, 14579–14584.
5. Williams, J.W. and Morrison, J.F. (1979) *Meth. Enzymol.*, **63**, 437–467.
6. Reimann, E.M. and Beham, R.A. (1983) *Meth. Enzymol.*, **99**, 51–55.
7. Olsen, S.R. and Uhler, M.D. (1989) *J. Biol. Chem.*, **264**, 18662–18666.
8. Cook, P.F., Neville, M.E., Jr., Vrana, K.E., Hartl, F.T. and Roskoski, R.R., Jr. (1982) *Biochemistry*, **21**, 5794–5799.
9. Bailey, J.E. (1966) Ph.D. thesis, London University, cited in *Practical Handbook of Biochemistry and Molecular Biology* (Fasman, G.D., Ed.), pp. 80–81. Boca Raton: CRC Press.
10. Bock, R.M., Ling, N.-S., Morell, S.A. and Lipton, S.H. (1956) *Arch. Biochem. Biophys.*, **62**, 253–264.
11. Peters, K.A., Demaille, J.G. and Fischer, E.H. (1977) *Biochemistry*, **16**, 5691–5697.
12. Shoji, S., Ericsson, L.H., Walsh, K.A., Fischer, E.H. and Titani, K. (1983) *Biochemistry*, **22**, 3702–3709.
13. Cleland, W.W. (1979) *Meth. Enzymol.*, **63**, 103–138.
14. Cheng, H.-C., Van Patten, S.M., Smith, A.J. and Walsh, D.A. (1985) *Biochem. J.*, **231**, 655–661.
15. Knighton, D.R., Zheng, J., Ten Eyck, L.F., Xuong, N.-h., Taylor, S.S. and Sowadski, J.M. (1991) *Science*, **253**, 414–420.
16. Scott, J.D., Fischer, E.H., Demaille, J.G. and Krebs, E.G. (1985) *Proc. Nat. Acad. Sci. U.S.A.*, **82**, 4379–4383.
17. Beavo, J.A., Bechtel, P.J. and Krebs, E.G. (1974) *Proc. Nat. Acad. Sci. U.S.A.*, **71**, 3580–3583.
18. Ashby, C.D. and Walsh, D.A. (1973) *J. Biol. Chem.*, **248**, 1255–1261.
19. Walsh, D.A. and Ashby, C.D. (1973) *Rec. Prog. Horm. Res.*, **29**, 329–359.
20. Zoller, M.J., Yonemoto, W., Taylor, S.S. and Johnson, K.E. (1991) *Gene*, **99**, 171–179.
21. Uhler, M.D. and McKnight, G.S. (1987) *J. Biol. Chem.*, **262**, 15202–15207.