The Catalytic Domain of *Acanthamoeba* Myosin I Heavy Chain Kinase

I. IDENTIFICATION AND CHARACTERIZATION FOLLOWING TRYPTIC CLEAVAGE OF THE NATIVE ENZYME

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The actin-activated Mg\(^{2+}\)-ATPase activities of the myosin I isoenzymes from *Acanthamoeba castellanii* are greatly increased by phosphorylation catalyzed by myosin I heavy chain kinase (MIHC kinase), a monomeric 97-kDa protein whose activity is greatly enhanced by acidic phospholipids and by autophosphorylation of multiple sites. In this paper, we show that the 35-kDa COOH-terminal fragment obtained by trypsin cleavage of maximally activated, autophosphorylated kinase retains the full activity and two to three of the autophosphorylation sites of the native enzyme. Other autophosphorylation sites occur in the middle third of the native enzyme. A tryptic cleavage site within the 35-kDa region is protected in phosphorylated kinase but is readily cleaved in unphosphorylated kinase producing catalytically inactive 25- and 11-kDa fragments from the NH\(_2\)- and COOH-terminal ends, respectively, of the 35-kDa peptide. This implies that the conformation around the "25/11" cleavage site changes upon phosphorylation of the native enzyme. The position of this site corresponds to the activation loop of protein kinase A (see the accompanying paper: Brzeska, H., Szczepanowska, J., Hoey, J., and Korn, E. D. (1996) *J. Biol. Chem.* 271, 27056–27062). Exogenously added MIHC kinase phosphorylates the 11-kDa fragment, but not the 25-kDa fragment, indicating that the phosphorylation sites of the 35-kDa catalytic fragment are located within the COOH-terminal 11 kDa. The accompanying paper describes the cloning, sequencing, and expression of a fully active 35-kDa catalytic domain.

Myosin I heavy chain kinase phosphorylates the three myosin I isoenzymes from *Acanthamoeba castellanii* and activates their actin-dependent Mg\(^{2+}\) -ATPase (for summary, see Ref. 1) and in vitro motility (2–4) activities. The phosphorylated Ser or Thr at this site are yeast (11), *Aspergillus* (12) and four *Dictyostelium* (13, 14) myosins I (for review, see Refs. 7 and 8). Two of the *Dictyostelium* isoenzymes have been shown to be activated in vitro by phosphorylation with *Acanthamoeba* MIHC (15, 16), and recently, a MIHC kinase similar to the *Acanthamoeba* kinase has been purified from *Dictyostelium* (17).

*Acanthamoeba* MIHC kinase is a 97-kDa monomer whose activity is regulated in vitro by several factors that have important physiological roles for other kinases (for review, see Ref. 8). Highly purified MIHC kinase autophosphorylates (independently) slowly; incorporation of 7–8 mol of phosphate/mol of kinase enhances its activity over 50-fold (18), and phosphorylation can reach 16 mol/mol. Association of MIHC kinase with acidic phospholipids (18, 19) or isolated plasma membranes (20) enhances the rate of phosphorylation of myosin I and of autophosphorylation of kinase (~20-fold). These stimulatory effects of phospholipid are Ca\(^{2+}\)-independent but are inhibited by Ca\(^{2+}\)-calmodulin (21), probably because Ca\(^{2+}\)-calmodulin and acidic phospholipids compete for the same binding site on the kinase.

The roles and locations of the multiple autophosphorylation sites and how the different regulatory factors affect the conformation, and hence the activity, of the kinase molecule are not known. Previous studies involving limited proteolysis by chymotrypsin showed that binding of both acidic phospholipid and Ca\(^{2+}\)-calmodulin to MIHC kinase is greatly reduced by removal of the NH\(_2\)-terminal 7 kDa of the polypeptide (21, 22) and that a 54-kDa COOH-terminal fragment (F54) retains the full kinase activity and 3–4 of the ~11 phosphorylation sites of the native enzyme. Just as for the native MIHC kinase, the activity of F54 is greatly enhanced by autophosphorylation; however, the rate of autophosphorylation of F54 is the same in the presence and absence of phospholipid and comparable with that of native kinase in the presence of phospholipid (22). These results suggest the presence of at least two, sequentially separated, regulatory regions: the acidic phospholipid (plasma membrane)/Ca\(^{2+}\) -calmodulin-binding NH\(_2\) terminus and the autophosphorylatable, catalytic COOH terminus.

In this paper, we report that a 35-kDa fragment obtained by tryptic proteolysis of fully phosphorylated MIHC kinase retains complete catalytic activity and three phosphorylation sites. The conformation of the 35-kDa region depends on the state of kinase phosphorylation with an internal site accessible to trypsin only when the kinase is not phosphorylated. In the accom-

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§ The abbreviations used are: MIHC, myosin I heavy chain; AEBSF, 4-(2-aminoethyl)-benzenesulfonyl fluoride; F, proteolytic fragment (with molecular mass in kDa following the F); PAK, p21-activated kinase; PKA, CAMP-dependent protein kinase; PAGE, polyacrylamide gel electrophoresis.
Fig. 1. Effect of phosphorylation on trypsin digestion of MIHC kinase. Unphosphorylated and $^{32}$P-labeled phosphorylated MIHC kinase (0.16 mg/ml) were incubated with trypsin (6 μg/ml) at 20°C in storage buffer diluted 1:2. Aliquots were removed at the indicated times, mixed with SDS sample buffer containing phenylmethylsulfonyl fluoride (final concentration, 1 mM), and analyzed by SDS-PAGE. An additional sample was taken from the unphosphorylated myosin digest at 60 min, trypsin-chymotrypsin inhibitor (25 μg/ml) added, and the mixture incubated with $[^{32}P]ATP$ for 15 min at 30°C under conditions for kinase autophosphorylation (lane 60 + ATP). Autophosphorylation was stopped by SDS sample buffer containing phenylmethylsulfonyl fluoride. Coomassie Blue-stained 11.5% acrylamide gels of the digests of unphosphorylated and phosphorylated kinase and the autoradiograms of the $^{32}$P-labeled digests are shown. The autoradiogram of lane 60 + ATP was a short exposure time. Longer exposure times show phosphorylation of F36 and F38 (see Fig. 5, lane g).

**RESULTS**

The State of Kinase Phosphorylation Affects the Rate and Final Products of Proteolysis—Unphosphorylated MIHC kinase (97 kDa) was rapidly cleaved by trypsin to four major fragments of approximate masses of 90, 66, 60, and 11 kDa and three minor fragments of 75, 45, and 35 kDa (Fig. 1, Coomassie, 2 and 5 min). By 30 min, all of these fragments had almost disappeared, except for the 11-kDa fragment which became stronger, and a doublet at 36 and 38 kDa and a prominent band at 25 kDa had appeared. The 25- and 11-kDa fragments were resistant to further proteolysis and became the major products by 60 min.

Trypsin cleavage of fully phosphorylated kinase, which, as reported previously (18), had a higher apparent mass by SDS-PAGE (107 kDa) than the unphosphorylated kinase, produced a similar pattern of large fragments (90, 75, 66, and 60 kDa) at short incubation times (Fig. 1, Coomassie, 2 and 5 min), but of slightly slower electrophoretic mobilities as expected for phosphorylated peptides. However, bands corresponding to the 45- and 11-kDa fragments from unphosphorylated kinase were not present (Fig. 1, 2 and 5 min). At longer digestion times of the phosphorylated kinase, a diffuse doublet of 36 and 38 kDa appeared (which is seen more clearly by autoradiography, Fig. 1). $^{32}$P-labeled protein standards.

Tryptic fragments were separated by SDS-PAGE, transferred to Immobilon PSQ membrane (Millipore), and sequenced on an Applied Biosystem model 470A sequenator equipped with on-line model 120A phosphorothiohydantoin analyzer (22). The transfer buffer was 25 mM Tris, 10 mM glycine, pH 8.3, 0.01% NaN₃ at 20°C. Kinase activity was measured (25) using as substrate synthetic peptide PC9, which corresponds to the phosphorylation site of myosin IC. All kinase assays were performed in buffer containing 2.5 mM $[^{32}P]ATP$ (20–40 cpm/pmol), 3.5 mM MgCl₂, 50 mM imidazole, pH 7.0, 1 mM EGTA, and 0.2 mg/ml of bovine serum albumin at 20°C for 1 min with a PC9 concentration of 0.2 mM. MIHC kinase and its tryptic cleavage fragments were autophosphorylated in the same buffer at 30°C with an ATP specific activity of 200–400 cpm/pmol. Proteins were separated by SDS-PAGE and incorporation of $^{32}$P was measured by liquid scintillation counting of solubilized, excised bands (22), or by scanning autoradiograms of gels which included $^{32}$P-labeled protein standards.

A polyclonal antibody (α11K) against a synthetic peptide corresponding to the NH₂-terminal sequence of F11 (ASVVGTYYWMAPFVV, see “Results,” synthesized using the MAP resin technology to increase the immunogenic response) was raised in rabbits by Research Genetics; its titer against synthetic peptide was greater than 1,500,000 by enzymelinked immunosorbent assay. For immunoblots, proteins and peptide fragments were separated by SDS-PAGE transferred to nitrocellulose and incubated with antibodies. Goat anti-rabbit peroxidase-conjugated IgG (Calbiochem) was used as second antibody.

**MATERIALS AND METHODS**

MIHC kinase was purified as described previously (21, 24) and stored in kinase storage buffer (20 mM Tris, pH 7.5, 50 mM KCl, 1 mM dithiothreitol, 0.01% Na₃) at −20°C. Kinase activity was measured (25) using as substrate synthetic peptide PC9, which corresponds to the phosphorylation site of myosin IC. All kinase assays were performed in buffer containing 2.5 mM $[^{32}P]ATP$ (20–40 cpm/pmol), 3.5 mM MgCl₂, 50 mM imidazole, pH 7.0, 1 mM EGTA, and 0.2 mg/ml of bovine serum albumin at 20°C for 1 min with a PC9 concentration of 0.2 mM. MIHC kinase and its tryptic cleavage fragments were autophosphorylated in the same buffer at 30°C with an ATP specific activity of 200–400 cpm/pmol. Proteins were separated by SDS-PAGE and incorporation of $^{32}$P was measured by liquid scintillation counting of solubilized, excised bands (22), or by scanning autoradiograms of gels which included $^{32}$P-labeled protein standards.

Tryptic fragments were separated by SDS-PAGE, transferred to Immobilon PSQ membrane (Millipore), and sequenced on an Applied Biosystem model 470A sequenator equipped with on-line model 120A phosphorothiohydantoin analyzer (22). The transfer buffer was 25 mM Tris, 192 mM glycine, pH 8.3, 0.01% SDS.

A polyclonal antibody (α11K) against a synthetic peptide corresponding to the NH₂-terminal sequence of F11 (ASVVGTYYWMAPFVV, see “Results,” synthesized using the MAP resin technology to increase the immunogenic response) was raised in rabbits by Research Genetics; its titer against synthetic peptide was greater than 1,500,000 by enzymelinked immunosorbent assay. For immunoblots, proteins and peptide fragments were separated by SDS-PAGE transferred to nitrocellulose and incubated with antibodies. Goat anti-rabbit peroxidase-conjugated IgG (Calbiochem) was used as second antibody.

The NH₂-terminal amino acid sequences of the 90-, 66-, 60-, and 38-kDa fragments derived from unphosphorylated kinase were the same as the NH₂-terminal sequences of the corresponding phosphorylated fragments (Table I). Thus, the latter were almost certainly the phosphorylated counterparts of the former. On the assumption that this was most likely also true for the fragments that were not sequenced, all of the proteolytic fragments from both phosphorylated and unphosphorylated kinase are hereinafter referred to by the apparent masses of the unphosphorylated fragments.

Phosphorylated F66 and F60 were consistently degraded much more slowly than unphosphorylated F66 and F60 and persisted until at least 90 min (Fig. 1); a slightly slower rate of
NH$_2$-terminal sequences of the tryptic fragments of myosin I heavy chain kinase

Unphosphorylated and phosphorylated MIHC kinase were digested with trypsin and the peptide fragments separated by SDS-PAGE, as shown in Fig. 1, and transferred to Immobilon-P. The membrane was stained with Ponceau S, and the appropriate bands were excised and placed directly in the sequenator (see "Materials and Methods"). F90, F66, and F60 were from 2-min digestions, and F38, F35, F25, and F11 were from 60-min digestions. The amount of each peptide was estimated by Coomassie Blue-staining of a parallel gel. As judged by residual material detectable by Coomassie Blue, essentially 100% of all peptides were transferred to Immobilon-P. The estimated amounts of Immobilon-bound peptides were: F90, unphosphorylated, 25 pmol; phosphorylated, 48 pmol; F66, unphosphorylated, 32 pmol; phosphorylated, 48 pmol; F60, unphosphorylated, 32 pmol; phosphorylated, 16 pmol; F35, phosphorylated, 100 pmol; F25, unphosphorylated, 100 pmol; F11, unphosphorylated, 100 pmol. The amounts of unphosphorylated and phosphorylated F38 were too low for reliable quantification.

| Cycle | F90 | F66 | F60 | F38 | F35 | F25 | F11 |
|-------|-----|-----|-----|-----|-----|-----|-----|
|       | −P  | +P  | −P  | +P  | −P  | +P  | −P  | +P  |
| 1     | Glu | Glu | Glu | Glu | Glu | Glu | Ala | Ala | Ala |
| 2     | Met | Met | Gln | Gln | Leu | Leu | Gly | Gly | Ser |
| 3     | His | His | Gln | Gln | Pro | Pro | Glu | Glu | Val |
| 4     | Ile | Ile | Pro | Pro | Pro | Pro | Ala | Ala | Val |
| 5     | Gly | Gly | Glu | Glu | XXX | XXX | Glu | Glu | Pro |
| 6     | Phe | Phe | Pro | Pro | Pro | Pro | Pro | Pro | Thr |
| 7     | Asp | Asp | Gln | Gln | Gln | Gln | Gln | Pro | Thr |
| 8     | Ser | Ser | Gln | Gln | Ala | Ala | Gln | Ala | Tyr |
| 9     | Gln | Gln | Ala | Ala | Ala | Ala | Ala | Ala | Trp |
| 10    | Thr | Thr | Pro | Pro | Asn | Asn | Ala | Ala | Met |
| 11    | Gly | Gly | Leu | Leu | Gly | Gly | Pro | Pro | Ala |
| 12    | Thr | Thr | XXX | XXX | Ala | Ala | Gln | Gln | Pro |
| 13    | Phe | XXX | Pro | Pro | Pro | Pro | Pro | Pro | Ala |
| 14    | Glu | Glu | Pro | Pro | Pro | Met | Ala | Ala | Val |
| 15    | Leu | Leu | Pro | Pro | Pro | Pro | Arg | Arg | Val |
| 16    |     |     |     |     |     |     |     |     |     |
| 17    |     |     |     |     |     |     |     |     |     |
| 18    |     |     |     |     |     |     |     |     |     |
| 19    |     |     |     |     |     |     |     |     |     |
| 20    |     |     |     |     |     |     |     |     |     |
| 21    |     |     |     |     |     |     |     |     |     |
| 22    |     |     |     |     |     |     |     |     |     |
| 23    |     |     |     |     |     |     |     |     |     |
| 24    |     |     |     |     |     |     |     |     |     |
| 25    |     |     |     |     |     |     |     |     |     |
| 26    |     |     |     |     |     |     |     |     |     |

* After digestion for 30 min, a second sequence identical to that of F66 appeared in the digest of unphosphorylated kinase suggesting that a second "F60" was formed by COOH-terminal cleavage of F66. This is likely the same fragment previously seen under slightly different digestion conditions (22).

Degradation was also observed for phosphorylated F90. There was no counterpart of phosphorylated F35 in the final tryptic products of unphosphorylated kinase. Conversely, tryptic digests of phosphorylated kinase never contained F25 or F11 that were the major stable products of tryptic digestion of unphosphorylated kinase. Although the relative amounts of the larger fragments varied in different experiments, the final products were always the same irrespective of the length of the digestion and with up to eight times more trypsin, i.e. F35 was always obtained from phosphorylated kinase and F25 and F11 from unphosphorylated kinase.

**Alignment of Tryptic Fragments of MIHC Kinase**—The different NH$_2$-terminal sequences of phosphorylated F90, F66, and F60 (Table I) suggest that they were derived from MIHC kinase by progressive cleavages at the NH$_2$-terminus; each may, or may not, extend to the COOH terminus of the native kinase (Fig. 2, lower). The NH$_2$-terminal sequence of F38 (Table I) was the same as that of F66, which indicates that F38 was derived from the NH$_2$ terminus of F66 (Fig. 2, lower left). This line of reasoning makes it most likely that F35, the final, stable digestion product of phosphorylated kinase whose NH$_2$-terminal sequence differed from those of all of the larger fragments (Table I), was derived from the COOH-terminal end of MIHC kinase (Fig. 2, lower left). The NH$_2$-terminal sequence of unphosphorylated F25 was identical to that of phosphorylated F35 and the NH$_2$-terminal sequence of unphosphorylated F11 differed from those of all of the other fragments of phosphorylated or unphosphorylated kinase (Table I). Therefore, it seems highly likely that F25 and F11 represented the NH$_2$- and COOH-terminal segments, respectively, of F35 (Fig. 2, lower right).

The assignments based on the NH$_2$-terminal sequences were supported by immunobots of SDS-PAGE gels (Fig. 3). Antibody α11K, which was raised against a 15-residue synthetic peptide corresponding to the NH$_2$-terminal sequence of F11, recognized F35 (Fig. 3, lane d) and F11 (Fig. 3, lane b), but not F25 (Fig. 3, lane b), consistent with F11 originating from the COOH-terminal region of F35. The antibody also recognized intact kinase (Fig. 3, lanes a and c, traces of F90 (Fig. 3, lanes b), F66 and F60 (Fig. 3, lanes b and d). These latter results support the conclusion that F90, F66, and F60 were produced by progressive cleavages at the NH$_2$ terminus of MIHC kinase and contained most, if not all, of the COOH-terminal 35-kDa region of the kinase.

Both F66 and F60 were more resistant to proteolysis when derived from phosphorylated kinase, but the most striking effect of phosphorylation was the difference in the final digestion products, i.e. COOH-terminal F35 from phosphorylated kinase and F25 and F11 from unphosphorylated kinase. Thus, phosphorylation strongly inhibits proteolysis at a site about 11 kDa from the COOH terminus. More detailed analysis of the data in Figs. 1 and 3 provides additional evidence for the greater susceptibility to proteolysis of the COOH terminus of unphosphorylated kinase. For example, F45, which occurred early in the digestion of unphosphorylated kinase (Fig. 1, 2 and 5 min) but was not seen in the digestion of phosphorylated kinase, was not recognized by α11K (Fig. 3, lane b), indicating that it lacks the COOH-terminal 11-kDa region. Also, sequence data suggest that the COOH terminus of F66 derived from unphosphorylated kinase, but not of F66 derived from phosphorylated kinase, is cleaved during extensive incubation with trypsin (see Table I, Footnote a).
Enzymatic Activities of Trypsin Digestion Products—We reported previously (24) that chymotrypsin digestion produces a 54-kDa peptide from the COOH terminus of both phosphorylated and unphosphorylated MIHC kinase and that F54 retains full catalytic activity and approximately four autophosphorylation sites that must be phosphorylated for maximal enzymatic activity. As the 35-kDa tryptic peptide should be contained within the 54-kDa chymotryptic peptide, it was of interest to determine whether F35, F25, and/or F11 were catalytically active. This was difficult to determine in digestion mixtures such as those shown in Fig. 1, because even after prolonged incubations, these still contained a number of larger fragments that would be expected to have enzymatic activity. To avoid this problem, we employed a two-step digestion procedure, chymotrypsin followed by trypsin (Fig. 2, upper). As expected, chymotrypsin cleaved phosphorylated kinase (Fig. 4, lanes a) to F54 (Fig. 4, lanes b) which was cleaved by trypsin to F35 (Fig. 4) and unphosphorylated F54 to F35 to F38 (Fig. 4) and unphosphorylated F64 to F25 and F11 (Fig. 5). The phosphorylated fragments and intact kinase have slower electrophoretic mobilities than the corresponding unphosphorylated peptides (Fig. 1). Some of the fragments might lack a small part of the COOH terminus of the intact kinase.

![Phosphorylated Kinase](image1.png)

**Fig. 2.** Schematic representation of the major trypsin cleavage sites and one chymotrypsin cleavage site of phosphorylated and unphosphorylated MIHC kinase. Lower, alignment of trypsin fragments identified in Fig. 1. Trypsin cleaves both phosphorylated (left) and unphosphorylated MIHC kinase (right) to F90, F66, F60, and F38. The final, stable products of trypsin digestion are different: F35 for phosphorylated kinase (left) and F25 and F11 for unphosphorylated kinase (right). The origin of each fragment is deduced from its NH2-terminal sequence (Table I and text). It is not known whether the COOH terminus of F38 overlaps the NH2 terminus of F35 (and F25). The phosphorylated fragments and intact kinase have slower electrophoretic mobilities than the corresponding unphosphorylated peptides (Fig. 1). The kinase activities of F54 and F35 produced by sequential chymotrypsin and trypsin digestion were significantly higher than the activity of native, phosphorylated kinase (Table II). The apparently still higher activity of F35 obtained by direct trypsin digestion may reflect a contribution from the contaminating larger fragments. Because the specific activities were calculated back to the initial kinase concentration, assuming 100% conversion to the final digestion product, the specific activities calculated for the fragments may be underestimated, and therefore, F35 is probably at least 45% more active than native, phosphorylated kinase.

![Unphosphorylated Kinase](image2.png)

**Enzymatic Activities of Trypsin Digestion Products**—We reported previously (24) that chymotrypsin digestion produces a 54-kDa peptide from the COOH terminus of both phosphorylated and unphosphorylated MIHC kinase and that F54 retains full catalytic activity and approximately four autophosphorylation sites that must be phosphorylated for maximal enzymatic activity. As the 35-kDa tryptic peptide should be contained within the 54-kDa chymotryptic peptide, it was of interest to determine whether F35, F25, and/or F11 were catalytically active. This was difficult to determine in digestion mixtures such as those shown in Fig. 1, because even after prolonged incubations, these still contained a number of larger fragments that would be expected to have enzymatic activity. To avoid this problem, we employed a two-step digestion procedure, chymotrypsin followed by trypsin (Fig. 2, upper).

As expected, chymotrypsin cleaved phosphorylated kinase (Fig. 4, lanes a) to F54 (Fig. 4, lanes b) which was cleaved by trypsin to F35 (Fig. 4, lanes c) that had identical SDS-PAGE mobility as F35 obtained by direct trypsin cleavage of phosphorylated kinase (Fig. 4, lanes d). The only difference was that the F35 obtained by two-step cleavage, like its 54-kDa precursor, appeared as a closely spaced doublet (Fig. 4, lanes c), probably, as suggested previously (22), because of partial chymotryptic cleavage at the COOH terminus. The F35 obtained by single step trypsin cleavage was more heavily contaminated by F66, F60, and the F36/F38 doublet, as is most easily seen on the autoradiogram (Fig. 4, lanes d).

The kinase activities of F54 and F35 produced by sequential chymotrypsin and trypsin digestion were significantly higher than the activity of native, phosphorylated kinase (Table II). The apparently still higher activity of F35 obtained by direct trypsin digestion may reflect a contribution from the contaminating larger fragments. Because the specific activities were calculated back to the initial kinase concentration, assuming 100% conversion to the final digestion product, the specific activities calculated for the fragments may be underestimated, and therefore, F35 is probably at least 45% more active than native, phosphorylated kinase.

The analogous experiment with unphosphorylated kinase is shown in Fig. 5. Trypsin digestion of F54 of the initial chymotrypsin digestion (Fig. 5, lane b) produced an amount of F25 (Fig. 5, lane c) comparable with that obtained by direct trypsin cleavage of native kinase (Fig. 5, lanes e and f). However, only a relatively small amount of F11 was obtained in the two-step...
to determine their $^{32}\text{P}$ content (Table II). Other aliquots of all samples nase after digestion with trypsin only. Bandswere eluted from the gels lanes b Coomassie Blue-stained gel and its autoradiogram are shown. Above, the NH$_2$ terminus of F66 originates from a position sequence as F66 (Table I). Therefore, if, as we have deduced concentration, 0.5 mM) to stop the digestion. Trypsin (50 $^\circ$C to obtain F54. An aliquot was taken, and AEBSF was added (final additional 15 min before stopping the digestion by adding AEBSF to 0.5 mm (final concentration). As a control, another sample of phosphorylated MIHC kinase was digested just with trypsin (50 $\mu$g/ml) for 15 min at 20 $\circ$C. Aliquots of all samples were subjected to SDS-PAGE, and the Coomassie Blue-stained gel and its autoradiogram are shown. Lanes a, undigested phosphorylated kinase; lanes b, phosphorylated kinase after digestion with chymotrypsin only; lanes c, phosphorylated kinase after digestion with chymotrypsin and trypsin; lanes d, phosphorylated kinase after digestion with trypsin only. Bands were eluted from the gels to determine their $^{32}\text{P}$ content (Table II). Other aliquots of all samples were used for assays of kinase activity (Table II). See text for further details and interpretation.

digestion (Fig. 5, compare lane c with lanes e and f) perhaps, at least in part, because the 54-kDa fragment is probably a mixture of two peptides with identical NH$_2$ termini, but differing slightly at their COOH-terminal ends (22). Also, if F11 is stabilized by interaction with other peptides, it might be less stable to trypsin when produced from F54 in the two-step procedure than when it is produced directly from native kinase. As expected, the enzymatic activity of unphosphorylated kinase was quite low, while the activity of the F54 derived from it was equivalent to that of the F54 obtained from phosphorylated kinase (Table II). This is because, as shown previously (22), F54 autoprophosphorylates much more rapidly than native kinase (in the absence of phospholipid) and thus becomes fully active very early in the assay of its ability to phosphorylate substrate. No kinase activity was detected when the digestion mixture containing F25 derived from unphosphorylated F54 was incubated with PC9.

Location of Phosphorylation Sites—As mentioned, all of the products of trypsin digestion of phosphorylated kinase that were detected by Coomassie Blue were phosphorylated (Fig. 1). The strong intensity on the autoradiogram of the F36/F38 doublet relative to its staining by Coomassie Blue (Fig. 1, 30, 60, and 90 min; Fig. 4, lanes d) indicates that those fragments were highly phosphorylated. F38 has the same NH$_2$-terminal sequence as F66 (Table I). Therefore, if, as we have deduced above, the NH$_2$ terminus of F66 originates from a position about 31 kDa from the NH$_2$ terminus of the native kinase, the highly phosphorylated F38 would originate from approxi-

mately the middle third of the molecule.

The autoradiogram of the digest of phosphorylated kinase shows that F35, which is derived from the COOH-terminal third of the native kinase, is phosphorylated (Figs. 1 and 4). When a digestion mixture obtained by one-step cleavage of unphosphorylated kinase, in which F25 and F11 were the main components, was incubated with [$\gamma^{32}\text{P}]$ATP, F11 was phosphorylated and its electrophoretic mobility decreased (Fig. 1, 60 + ATP; Fig. 5, lane g), but $^{32}\text{P}$ was not incorporated into F25. As would be expected, F60 and the F60/F38 doublet were also labeled (Fig. 5, lane g). The same results were obtained when the digestion mixture was incubated with fully active, phosphorylated native kinase. F11 became heavily labeled with only traces of radioactivity incorporated into F25 (data not shown). Thus, the autoprophosphorylation site(s) in the COOH-terminal third of the kinase molecule are, most likely, exclusively in the COOH-terminal 11 kDa region. Only traces of $^{32}\text{P}$ were incorporated into F11 when the products of the two-step digestion of unphosphorylated kinase were incubated with [$\gamma^{32}\text{P}]$ATP (Fig. 5, lane d), in agreement with the observation that this mixture lacks significant kinase activity (Table II).

The phosphate contents of F54, F35, F25, and F11 were quantified from the experiments illustrated in Figs. 1, 4, and 5. Intact kinase was autoprophosphorylated to the extent of 16 mol/mol. F54 derived from autoprophosphorylated kinase retained about 4 mol and F35 about 3 mol of phosphate when obtained by one-step trypsin cleavage and about 1.5 mol when obtained by two-step cleavage (Table II). The amount of $^{32}\text{P}$ incorporated into F11 obtained by two-step cleavage was too small to measure, and F11 obtained by direct, one-step trypsin cleavage incorporated about 3 mol of phosphate/mol (Table II). Within the limits of these assays, then, it appears that about four of the autoprophosphorylatable sites of native kinase reside in the COOH-terminal 54-kDa region and three in the COOH-terminal F35, with essentially all of the latter being in the COOH-terminal F11 segment and none in the adjacent F25.

| Sample | Specific activity $\mu$mol/(min/mg) | Phosphorylation sites mol/mol |
|--------|-----------------------------------|-------------------------------|
| Phosphorylated Native kinase          | 12.4                           | 16                           |
| F54 (C)                               | 21.6                           | 4.4                          |
| F35 (C, T)                            | 17.3                           | 1.4                          |
| F35 (T)                               | 25.1                           | 2.7                          |
| Unphosphorylated Native kinase        | 1.1                            | 3.0                          |
| F54 (C)                               | 18.3                           | 3.4                          |
| F25 (C, T)                            | 0                              | 0                            |
| F11 (C, T)                            | 0                              | 0                            |
| F25 (T)                               | 0                              | 0                            |
| F11 (T)                               | 0                              | 0                            |

*F54, F35, etc. refer to proteolytic fragments of the indicated masses (by SDS-PAGE) obtained from native phosphorylated or unphosphorylated kinase (as indicated) by either chymotrypsin (C), trypsin (T), or chymotrypsin followed by trypsin (C, T) digestion.*

![FIG. 4. Preparation of the tryptic 35-kDa fragment (F35) from the chymotryptic phosphorylated 54-kDa fragment (F54). $^{32}\text{P}$-Labeled phosphorylated MIHC kinase (0.16 mg/ml) was digested in storage buffer diluted 1:2 with chymotrypsin (6 $\mu$g/ml) for 45 min at 20 $\circ$C to obtain F54. An aliquot was taken, and AEBSF was added (final concentration, 0.5 mM) to stop the digestion. Trypsin (50 $\mu$g/ml) was added to the rest of the sample and the incubation continued for an additional 15 min before stopping the digestion by adding AEBSF to 0.5 mM (final concentration). As a control, another sample of phosphorylated MIHC kinase was digested just with trypsin (50 $\mu$g/ml) for 15 min at 20 $\circ$C. Aliquots of all samples were subjected to SDS-PAGE, and the Coomassie Blue-stained gel and its autoradiogram are shown. Lanes a, undigested phosphorylated kinase; lanes b, phosphorylated kinase after digestion with chymotrypsin only; lanes c, phosphorylated kinase after digestion with chymotrypsin and trypsin; lanes d, phosphorylated kinase after digestion with trypsin only. Bands were eluted from the gels to determine their $^{32}\text{P}$ content (Table II). Other aliquots of all samples were used for assays of kinase activity (Table II). See text for further details and interpretation.
The mixture containing unphosphorylated F25 and small amount of F11 derived from the 35-kDa COOH terminus of unphosphorylated kinase by two step cleavage had no detectable kinase activity when assayed with synthetic peptide as substrate and did not autophosphorylate, although F11 could be phosphorylated by native kinase or large fragments that retained kinase activity. The simplest interpretation of these results is that cleavage of the 35-kDa catalytic domain at the "25/11" site disrupts the integrity of its catalytic site (Fig. 6).

The MIHC kinase catalytic domain (F35) obtained from fully phosphorylated native kinase contained 2.7 mol of phosphate/mol. F11 obtained from unphosphorylated kinase incorporated 3 mol of phosphate/mol when incubated with active kinase, whereas no phosphate was incorporated into F25. Therefore, despite some experimental limitations (e.g., the calculations of stoichiometry assume 100% recovery of digestion products, 1 mol/mol could reflect partial phosphorylation of more than 1 site, and peptides might be phosphorylated differently than the parent enzyme), these data strongly indicate that the catalytic domain of MIHC kinase contains two to three phosphorylation sites, all located within the COOH-terminal 11 kDa.

It is interesting that the 25/11 site is readily accessible to trypsin cleavage when MIHC kinase is unphosphorylated, but strongly protected in phosphorylated kinase. As discussed more extensively in the accompanying paper (23), the 25/11 trypsin cleavage site lies within a variable "linker" region between residues that are highly conserved in the ~370 kinases that have been sequenced (26–29). Many kinases are (auto)phosphorylated within the linker region, and this phosphorylation often affects kinase activity (for summary, see Refs. 28–30), presumably by inducing a conformational change. For example, the crystal structure of unphosphorylated, inactive MAP kinase ERK2 shows that the linker region partially blocks the substrate-binding site (31), and Cdk2 kinase (32) and PAK (30, 33) must be phosphorylated within this region for maximal activity. The change in susceptibility of MIHC kinase to trypsin cleavage described in this paper provides direct evidence for a phosphorylation-dependent conformational change in this functionally important loop and a conformational change in the linker region of cyclin-dependent kinase (Cdk2) was recently demonstrated by solving the crystal structures of the inactive (32) and partially activated (in complex with cyclin A (34)) forms (for summary, see Ref. 35).

As shown in the accompanying paper (23), MIHC kinase has a putative autophosphorylation site downstream from the 25/11 cleavage site. This is likely to be one of the sites that are phosphorylated in F11. By sequence analogy (23), the 25/11 cleavage site lies in a region that corresponds to the activation
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loop of PKA and the putative autophosphorylation site in the P+1 loop (29). Although, we were unable to determine the effect of phosphorylation on the activity of F35 (which contains F11), because we could not obtain this fragment in its unphosphorylated form, we have shown previously (22) that the activity of the COOH-terminal F54 fragment (which contains F35) is enhanced by autophosphorylation.

The NH2-terminal sequences of trypsin fragments F66, F60, and F55 (Table 1) and chymotrypsin fragments F60 and F54 (22) are all proline-rich, indicating that the middle third of MIHC kinase has a very high proline content (Fig. 6). Interestingly, proline-rich and polyacidic sequences separate the catalytic domain from the regulatory p21-binding domain of PAK (36), and PAK is activated by autophosphorylation within its proline-rich region as well as within the catalytic domain (37, 38). Although it is not known whether this region has a similar role in all kinases, the presence of phosphorylation sites within this region of MIHC kinase (Fig. 6) raises the possibility that phosphorylation might regulate the conformation of this region as well the conformation of the linker region. This possibility is strengthened by our observation that phosphorylation of MIHC kinase increases the resistance of this region to proteolysis. The sequence similarity of MIHC kinase to the PAK family is discussed in more detail in the accompanying paper (23).

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CLEAVAGE OF THE NATIVE ENZYME
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J. Biol. Chem. 1996, 271:27049-27055.
doi: 10.1074/jbc.271.43.27049

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