Demonstration of Three Distinct Calcium-binding Sites in Villin, a Modulator of Actin Assembly

Lyndal R. Hesterberg and Klaus Weber
From the Max Planck Institute for Biophysical Chemistry, D-3400 Goettingen, Federal Republic of Germany

Villin, a Ca\textsuperscript{2+}-modulated F-actin-binding protein (95,000 daltons) present in microvillus core filament bundles, has been shown to contain multiple Ca\textsuperscript{2+}-binding sites. \textsuperscript{40}Ca Hummel-Dreyer chromatography reveals the presence of two rapidly exchanging Ca\textsuperscript{2+}-binding sites with an apparent dissociation constant, \(K_d\), equal to \(4.6 \times 10^{-8}\) M. Use of the two proteolytically separable domains of the molecule revealed that one site is located on the 90,000-dalton core (apparent \(K_d\) = \(3.5 \times 10^{-8}\) M) while the second site is provided by the 8,800-dalton headpiece fragment (apparent \(K_d\) = \(7.4 \times 10^{-6}\) M). In addition villin displays a very slowly exchanging or nonexchangeable high affinity Ca\textsuperscript{2+}-binding site, which is situated in the core domain. Secondary structural predictions and a comparison of the amino acid sequence of headpiece with other known Ca\textsuperscript{2+}-binding proteins indicates one region suggestive of a Ca\textsuperscript{2+}-binding site, although headpiece seems not to exhibit a classical "EF-hand" Ca\textsuperscript{2+}-binding structure.

The role of calcium as an intracellular mediator of microfilament architecture and regulation has been an area of intense research during the last few years (for recent reviews see Refs. 1 and 2). The actin filament bundle supporting the plasma membrane extension of the microvilli present on intestinal epithelial cells provides a particularly interesting system. This structure can be isolated in a homogeneous membrane-free form and reveals in addition to actin at least four major associated proteins, of which three bind Ca\textsuperscript{2+} in the micromolar range (3-8). One of the striking properties of core filament bundles (isolated in the presence of EGTA) is their disintegration once the free Ca\textsuperscript{2+} concentration reaches micromolar (3, 6, 7). Since calmodulin-freed bundles (8), as well as bundles reconstituted in vitro from villin and actin (7, 10) show the same property, interest has focused on a possible calcium-dependent transition of villin (\(M_f = 95,000\)).

Villin displays two structurally and functionally distinct domains. Mild in vitro proteolysis separates an actin-binding headpiece (8,800 daltons) from a large core fragment (95,000 daltons) which displays villin's Ca\textsuperscript{2+}-dependent activities on in vitro actin assembly. Neither of the two fragments are capable of bundling actin (12-14).

The preceding paper (9) has shown that addition of Ca\textsuperscript{2+} induces a large change in the hydrodynamic properties of villin. The conformational change is due to an increase in the frictional coefficient of villin and not to dimerization. Since this change was not detected with the isolated core (9), the results raise the question of the role of core and headpiece in Ca\textsuperscript{2+} binding.

Using Ca\textsuperscript{2+}-binding studies we have now identified multiple sites on the villin molecule. In addition to a nonexchangeable site, we document two rapidly exchanging sites, one on headpiece and one on core. We discuss the possible functional significance of the latter two sites and correlate the headpiece Ca\textsuperscript{2+}-binding site to other sites established for various Ca\textsuperscript{2+}-binding proteins.

EXPERIMENTAL PROCEDURES

Isolation of Villin, Core, and Headpiece--Villin was isolated using the modifications on the standard procedures (3, 12) given before (9). Villin was digested with V-8 protease. Core and headpiece were isolated as described (13). Protein concentrations were determined by absorbance at 290 nm using an absorptivity of 0.86 liter/g-cm for headpiece, 1.20 liters/g-cm for core (13), and 1.28 liters/g-cm for villin in EGTA (9).

Circular Dichroism—CD measurements were made on a calibrated Jobin Yvon Mark V autocorgraph spectropolarimeter as before (15). Measurements were made using a 0.25-cm or 0.4-cm pathlength cell with protein solutions ranging from 150 \(\mu\)g/ml to 1.0 mg/ml at 20.0 \(\pm 0.1\) °C over a range from 350 to 200 nm. Stock solutions of core and headpiece were prepared by passing them over a pre-equilibrated Sephadex G-25 column in 10 mM Tris, 10 mM NaCl, pH 8.5, and either 50 \(\mu\) M Ca\textsuperscript{2+} or 50 \(\mu\) M EGTA. All solutions were centrifuged and Milipore-filtered prior to use. Spectra were repeated using different preparations of core and headpiece.

Ca\textsuperscript{2+}-binding Studies—Nalgene beakers, tubes, and bottles were used throughout to avoid contamination with Ca\textsuperscript{2+} from laboratory glassware. Absolute concentration of Ca\textsuperscript{2+} in the various buffers and solutions was monitored using a UNICAM SP90B series 2 atomic absorption spectrophotometer with Chelex-treated distilled deionized H\textsubscript{2}O as the reference.

\(\text{Ca}^{2+}\) binding to villin, core, and headpiece was studied using the chromatographic method of Hummel and Dreyer (11). \(\text{Ca}^{2+}\) was purchased from New England Nuclear. A Sephadex G-25 fine column (0.6 \(\times\) 20 cm) at room temperature, 23 \(\pm 2\) °C, was equilibrated with the desired buffer. The flow rate was controlled with a Pharmacia F-3 peristaltic pump.

In order to obtain villin and core free of exchangeable Ca\textsuperscript{2+}, approximately 1 ml protein solution, stored in an EGTA-containing solution, was dialyzed for 48 h against 1 liter of Ca\textsuperscript{2+}-free H\textsubscript{2}O (3 changes). The protein solution was centrifuged and the integrity of the proteins monitored with sodium dodecyl sulfate-polyacrylamide gel electrophoresis prior to use. The protein was equilibrated in the column buffer before loading. Using this technique, the protein was eluted from the column in 20-30 min. 0.5-Ml fractions were collected, mixed with liquid scintillation fluid obtained from Baker Chemicals, and counted using a Beckman LS-220 liquid scintillation system. The ratio of Ca\textsuperscript{2+} to \(\text{Ca}^{2+}\) for each buffer could be calculated. \(\text{Ca}^{2+}\) binding was determined using the area of the radioactive peak corresponding to the \(\text{Ca}^{2+}\) bound to the protein according to the method of Levi et al. (17). The data were then plotted with Scatchard plots, curves fit.
Three Ca\textsuperscript{2+}-binding Sites in Villin

with least squares analysis ($R^2 \geq 0.95$), and the stoichiometry and binding constants calculated. An average error of ±10% in the binding constants is estimated due to experimental error and temperature variations.

Headpiece as purified by the method of Glenney et al. (13) is essentially Ca\textsuperscript{2+} free (<5 × 10\textsuperscript{-7} M) and was used without further purification. The buffer used to determine Ca\textsuperscript{2+} binding at pH 8.5 was 50 mM Tris, 50 mM NaCl, 0.1 mM dithiothreitol, 1.0 mM MgCl\textsubscript{2} plus the desired level of Ca\textsuperscript{2+} and Ca\textsuperscript{4+}. At pH 7.0 and buffer system remained the same.

Villin Renaturation—Villin was denatured and renatured using essentially the method of Hager and Burgess (18). 100 ml of villin (22 mg/ml) was dialyzed into 6 M guanidine-HCl, 50 mM Tris, pH 8.5, at room temperature for 24 h to remove free Ca\textsuperscript{2+}. The villin was then dialyzed into Ca\textsuperscript{2+}-free (<5 × 10\textsuperscript{-7} M Ca\textsuperscript{2+}) 6 M guanidine-HCl, 50 mM Tris, pH 8.5, as before, in order to remove all EGTA. Ca\textsuperscript{2+} to ~5 × 10\textsuperscript{-4} M was added to the protein solution and allowed to equilibrate for 1 h, at which time the solution was diluted 50-fold with Ca\textsuperscript{2+}-free 50 mM Tris, 50 mM NaCl, pH 8.5. The protein was permitted to renature for 18 h at room temperature. The protein-Ca\textsuperscript{2+} solution was dialyzed against a 2000-fold volume of pH 8.5 buffer containing 50 mM Tris, 50 mM NaCl, 0.1 mM dithiothreitol, 1.0 mM MgCl\textsubscript{2}, 100 mM CaCl\textsubscript{2} for 24 h. Aliquots of dialysate and buffer were removed and the Ca\textsuperscript{2+} counted with a liquid scintillation system (Beckman), and the total concentration of Ca\textsuperscript{2+} was determined using atomic absorption spectroscopy.

RESULTS

Villin Contains Two Rapidly Exchanging Calcium Sites

Located on the Headpiece and the Core—Ca\textsuperscript{2+}-binding experiments on villin, core, and headpiece were undertaken to define the stoichiometry and affinity of the Ca\textsuperscript{2+} interactions. The results obtained by the Hummel-Dreyer chromatography at pH 8.5 are shown in Fig. 1. Fig. 1 shows a typical elution profile. The peak of Ca\textsuperscript{45} coincides with the protein elution, in this case headpiece, and is separated from the resultant trough. This profile is indicative of a system in equilibrium and not suffering from protein overloading (22, 23). Ca\textsuperscript{45} binding was observed over a range from 1 × 10\textsuperscript{-7} M Ca\textsuperscript{2+} to 20 × 10\textsuperscript{-7} M Ca\textsuperscript{2+} in 50 mM Tris, 50 mM NaCl, 0.1 mM dithiothreitol, 1.0 mM MgCl\textsubscript{2}, pH 8.5, using a series of protein-loading concentrations. No effect of protein concentration dependence was observed on the binding parameters nor did the elution profiles show trailing shoulders of unbound ligand. Thus it is unlikely that this system exhibits ligand-mediated association as discussed by Cann and Hinman (23). The Scatchard plots are shown in Fig. 2 and are linear within the range of Ca\textsuperscript{2+} concentration studied. The respective stoichiometries and binding constants are summarized in Table I and reveal two apparent Ca\textsuperscript{2+}-binding sites in rapid exchange with the solvent, one of which is observed on villin core and the other on headpiece. The apparent binding constants are very similar, 3.5 and 7.4 μM, and are not influenced by the presence of 1 mM MgCl\textsubscript{2}.

![Fig. 1. Results of Hummel-Dreyer chromatography. Representative elution profile for the measurement of bound \textsuperscript{45}Ca. Elution profile is obtained from the counts per min of \textsuperscript{45}Ca of the individual 0.5 ml fractions of effluent.](image)

![Fig. 2. Scatchard plots for the binding of Ca\textsuperscript{2+} by villin, core, and headpiece.](image)

**TABLE I**

| Stoichiometry | Apparent $K_a$ (M) |
|---------------|-------------------|
| Villin        | 2.05              | 4.6 × 10\textsuperscript{-9} |
| Core          | 0.86              | 3.5 × 10\textsuperscript{-9} |
| Headpiece     | 1.10              | 7.4 × 10\textsuperscript{-9} |

*Buffer: 50 mM Tris, 50 mM NaCl, 0.1 mM dithiothreitol, 1.0 mM MgCl\textsubscript{2}, pH 8.5.*

Villin Contains a Slowly Exchanging Ca\textsuperscript{2+} Site—Although villin reveals two rapidly exchangeable Ca\textsuperscript{2+} sites in solution, this result cannot provide information on the possible existence of any slowly exchanging or nonexchangeable Ca\textsuperscript{2+} sites. The stoichiometry of slowly exchanging Ca\textsuperscript{2+} was observed in native villin using a different approach. Duplicate samples of villin at 3.6, 2.1, and 1.0 mg/ml were dialyzed 24 h against 10 mM Tris and 1 mM EGTA, pH 8.0, to remove any exchangeable Ca\textsuperscript{2+}. The protein was then exhaustively dialyzed against a 2000-fold volume of Ca\textsuperscript{2+}-free 10 mM Tris, 50 mM NaCl, 0.1 mM dithiothreitol, 1.0 mM MgCl\textsubscript{2}, pH 8.5. Villin was permitted to renature for 18 h at room temperature. The protein-Ca\textsuperscript{2+} solution was dialyzed against a 2000-fold volume of pH 8.5 buffer containing 50 mM Tris, 50 mM NaCl, 0.1 mM dithiothreitol, 1.0 mM MgCl\textsubscript{2}, 100 mM CaCl\textsubscript{2} for 24 h. Aliquots of dialysate and buffer were removed and the Ca\textsuperscript{2+} counted with a liquid scintillation system (Beckman), and the total concentration of Ca\textsuperscript{2+} was determined using atomic absorption spectroscopy. The Ca\textsuperscript{2+} concentration of the dialysis buffer was ≤0.5 × 10\textsuperscript{-7} M Ca, the limit of the atomic absorption spectrometer's resolution, while the Ca\textsuperscript{2+} concentration present in the dialyzed villin solution was 38 × 10\textsuperscript{-8} M for the highest protein concentration used. The concentration of Ca\textsuperscript{2+} measured represented a molar ratio of 1.2 Ca\textsuperscript{2+} atoms per villin monomer when averaged for the three villin concentrations tested. This result indicates that one very slowly exchanging, i.e. tightly bound, Ca\textsuperscript{2+} remains associated with native villin.

Villin core was studied in a similar manner to observe if the slowly exchanging site is located in the core domain or if it lost upon digestion. Samples of core at 2.7 mg/ml after having been dialyzed in the identical manner as villin were monitored using atomic absorption spectroscopy. The Ca\textsuperscript{2+} concentration of the dialysis buffer was ≤0.5 × 10\textsuperscript{-7} M Ca, while the Ca\textsuperscript{2+} concentration present in the dialyzed core solution was 33 μM. This represents a molar ratio of 1.2 Ca\textsuperscript{2+} atoms/core monomer and demonstrates that the slowly exchanging Ca\textsuperscript{2+} is retained in the core domain.

To further test this hypothesis, villin was denatured in 6 M guanidine-hydrochloride as outlined under “Experimental Procedures” and then renatured in the presence of 5 × 10\textsuperscript{-8} Ca\textsuperscript{2+}.
The characteristic secondary structure observed for calmodulin-like Ca\textsuperscript{2+}-binding proteins consists of the “EF-hand” architecture (helix-loop-helix) (28). Does villin headpiece also exhibit such a structure? The secondary structure of headpiece was predicted using the method of Chou and Fasman (19, 20). The results shown in Table II indicate a predominantly β-sheet structure on the NH\textsubscript{2} terminus interrupted by a turn and a short segment of random structure. The region exhibiting the putative sequence homology would be predicted to contain two extremely strong β-turns extending from amino acids 29–32 and 34–37, followed by a stretch of random structure. The region between residues 44 and 32, inclusive, is particularly interesting, as the average predicted values for helix and β-sheet for this region are 1.18 and 1.20, respectively. As discussed by Chou and Fasman (21), such variable regions are capable of undergoing conformational changes depending on environmental conditions.

The secondary structure of headpiece, however, is not characteristic of the “EF-hand” architecture, although the loop structure at the possible Ca\textsuperscript{2+} site seems similar to those observed in other Ca\textsuperscript{2+}-binding proteins (15). Tufty and Kretsinger (28) have proposed a test to recognize “EF-hand” (helix-loop-helix) regions based on the amino acid sequence of a protein. If a minimum of 10 out of 16 critical structure forming and liganding residues can be aligned to a 29-residue test sequence, the region is considered a calcium binding “EF-hand”. When this test is applied to villin headpiece no sequence can be found which contains at least 10 of 16 of the test residues. The sequence which scores the highest (6 of 16) is shown in Table III. Since headpiece is a calcium-binding protein, it may contain a variant binding site. Table III also contains the sequences for the crystallographically determined calcium-binding sites of carp parvalbumin, from which the “10 of 16” test was derived, and vitamin D-dependent calcium-binding protein (ICaBP). The structure of ICaBP, however, includes a variant Ca\textsuperscript{2+}-binding site, residues 11–32, which does not meet the “10 of 16” test requirements (25). Another

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig3}
\caption{Circular dichroism of villin core (A) and headpiece (B) in the presence of 50 μM EGTA (...) or 50 μM CaCl\textsubscript{2} (——) expressed as mean residue ellipticities in units of deg cm\textsuperscript{2} dmol\textsuperscript{-1}. Buffer conditions were 10 mM Tris, 10 mM NaCl, and either 50 μM EGTA or CaCl\textsubscript{2} at pH 8.5.}
\end{figure}
Three Ca$^{2+}$-binding Sites in Villin

**Table II**

The predicted secondary structures of villin headpiece

| PS | BB B B B B B B B B | V T F E T L P K T D | V L V V N T A A E D L | P |
|---|---------------------|---------------------|---------------------|---|
| EF | R R G V D P S R K E | N H L S D E D F P K | A O V F G M T R S A F | A N |
| | T R T T T T T T R R R R | R R R R R R R R | *** *** *** *** *** R | R |

**Table III**

Amino acid sequences of some calcium-binding proteins with a comparison to villin headpiece

The sequences are aligned to show the homologous calcium-binding regions. The residues underlined are either assumed to be involved in calcium coordination or known to do this by x-ray crystallographical analysis (for details see text). The standard amino acid abbreviations are provided in Table II.

| MCBP* | F A I D D Q R K S G F E I E D E L K L F Q F |
|---|---|
| CD | K A G Q D D G E G I G D V E T A L Y K |
| EF | T R T T T T T T T R R R R | R R R R R R R |
| ICaBP (bovine intestine, minor A) | K E A A K E G D H P N Q L S K E E L K L L L O | K F E E L D K R G D G E V S E F E P Q V L V K |

Villin headpiece

| S-100 PAPI-b (bovine brain) |
|---|
| D L P R Q V D P S R K N H L S D E F K A |

| S-100 PAPI-b (bovine brain) |
|---|
| R Q S G R E G D K H E L K K S E L K E L L |
| M E T L D S G D G C D F E Q F E M A F V A |

* Structure determined by x-ray crystallography.

**Discussion**

Villin, a Ca$^{2+}$-modulated protein, bundles actin filaments at Ca$^{2+}$ concentrations less than micromolar while limiting filament length at Ca$^{2+}$ greater than micromolar (3, 7, 10). Villin has also been shown to undergo a large Ca$^{2+}$-induced conformational change within this range of Ca$^{2+}$ concentrations (9). The two structural domains of villin, obtained through limited proteolysis, are a Ca$^{2+}$-independent F-actin binding headpiece (M$_r$ = 8,800) and a Ca$^{2+}$-sensitive core (M$_r$ = 87,000) which both nucleates actin and restricts filament length (13, 14). Core, however, is not capable of exhibiting the Ca$^{2+}$-induced hydrodynamic change observed in villin (9). The questions of how villin is able to perform both bundling and length restriction activities and how these are related to the observed conformational changes have been approached by this study which attempts to localize and describe the Ca$^{2+}$-binding sites on villin.

The role of the very slowly exchanging, tightly bound, Ca$^{2+}$ in villin is unknown, but may represent a structural site inaccessible to the solvent. As shown, the location of this structural site is on the 87,000-dalton core fragment. A similarity may exist between villin and certain Ca$^{2+}$-binding proteins whose structures have been crystallographically determined. Szebenyi et al. (25) reported the existence of a structural Ca$^{2+}$ site in vitamin D-dependent calcium-binding protein from bovine intestine, and Moews and Kretsinger (26) have identified the presence of a Ca$^{2+}$ site, inaccessible to solvent, in carp muscle calcium-binding parvalbumin. Thus, although this category of site is not without precedent in other Ca$^{2+}$-binding proteins it provides the first report in a protein known to exert Ca$^{2+}$-dependent modulation of actin filaments.

In contrast to preliminary equilibrium dialysis experiments in which villin was observed to display a single exchangeable Ca$^{2+}$-binding site with a $K_d = 2.5 \times 10^{-6}$ m at pH 7.3 (8), the results of the Hummel-Dreyer $^{45}$Ca-binding studies summarized here clearly reveal two rapidly exchanging Ca$^{2+}$ sites, one of which is located on core and the other on headpiece. The two sites, being located on different domains of the villin molecule are probably not identical, although the $K_d$ values obtained for each separately are reasonably close. The binding data from villin represent a sum of the stoichiometry of both nucleates actin and restricts filament length.
Ca\(^{2+}\)-binding site would control these activities. The headpiece fragment, by contrast, has exhibited no Ca\(^{2+}\)-dependent activities thus far as it seems to bind equally well to F-actin in the presence of EGTA or Ca\(^{2+}\) (13), but is required for F-actin cross-linking by villin in the absence of Ca\(^{2+}\).

Hydrodynamic data presented previously have shown that villin core is incapable of displaying the extensive hydrodynamic change observed in intact villin upon the addition of calcium (9). Nevertheless, core retains both Ca\(^{2+}\)-binding constant \(K_d = 3.5 \times 10^{-4}\) M, and Ca\(^{2+}\)-induced alterations in the secondary structure as observed in CD spectra. This evidence supports the concept that headpiece is involved in an essential role for the expression of the Ca\(^{2+}\)-induced conformational change of intact villin as observed in hydrodynamic changes. Upon addition of Ca\(^{2+}\) intact villin alters its sedimentation coefficient to become a distinctly more asymmetric molecule (9), while villin core does not show this property. This suggests that the headpiece domain must be repositioned relative to the core in the intact villin molecule to give rise to the observed increase in asymmetry. The exact mechanism by which this repositioning occurs cannot be elucidated by our solution studies, but secondary structural predictions of headpiece suggest one possible site of action within this domain, the "variable" region located at the COOH-terminal side of the proposed Ca\(^{2+}\)-binding loop. Although inherently speculative by nature, since limited by calculated secondary structure prediction methods and the lack of true three-dimen- sional structural evidence, such a hypothesis is interesting, since the proposed site may represent a structure in which the poly peptide backbone (see under "Results"). It must also be stated that while no direct evidence exists at this time to conclusively link the observed Ca\(^{2+}\)-dependent conformational changes to the known Ca\(^{2+}\)-dependent actin modulation activities of villin a similar range of Ca\(^{2+}\) concentrations is required to induce the changes in conformation (9) and to affect the stability of the microvillus actin filament bundle which contains villin as a structural component (3, 7, 8, 10).

Since two rapidly exchanging Ca\(^{2+}\)-binding sites have been observed the possibility exists that one is responsible for the Ca\(^{2+}\)-sensitive activities of villin that are retained by the core, nucleation, and filament length restriction, while the second would be responsible for the large conformational changes observed which require the headpiece domain. This hypothesis does not exclude the hinge mechanism as outlined in the previous paper (9), but rather provides additional insight by suggesting a complementary mechanism for the Ca\(^{2+}\) regulation of the conformational change. In such a model, the hinge mechanism would allow for cross-linking of actin filaments by villin in the absence of Ca\(^{2+}\) provided the actin-binding site located in the headpiece domain is in the proper orientation, i.e. when it is in a more compact conformation. Upon the binding of Ca\(^{2+}\), the relative position of the headpiece domain is shifted in relation to the core to a more asymmetric conformation, resulting in an orientation of the headpiece domain's actin-binding site that is no longer suitable for the cross-linking of F-actin filaments. This mechanism would have the advantage that it does not require Ca\(^{2+}\) sensitivity for headpiece binding to F-actin filaments, agreeing with previous reports (13). It would also allow for the possibility that the headpiece Ca\(^{2+}\) site is unrelated to the previously reported Ca\(^{2+}\)-dependent activities of core, but would still be essential for the Ca\(^{2+}\)-regulated transitions of the intact villin molecule.

Although the physical-chemical data collected thus far cannot allow one to determine the exact mechanism responsible for the regulation of villin's Ca\(^{2+}\)-sensitive bundling and sev ering activities, they clearly have required a more elaborate model for villin's activities. Nevertheless, the presence of three Ca\(^{2+}\)-binding sites together with the large Ca\(^{2+}\)-induced conformational change has provided a much clearer picture of villin's structure.

Acknowledgments—We acknowledge helpful discussions with Drs. J. R. Glenney, P. Matsudaira, and N. Geisler.

REFERENCES
1. Weeds, A. (1982) Nature 296, 811-816
2. Schliwa, M. (1981) Cell 25, 587-590
3. Bretscher, A., and Weber, K. (1980) Cell 20, 839-847
4. Bretscher, A., and Weber, K. (1980) J. Cell Biol. 86, 335-340
5. Mooseker, M. S., and Tilney, L. G. (1976) J. Cell Biol. 77, 725-743
6. Glenney, J. R., Jr., Kauflus, P., Matsudaira, P., and Weber, K. (1981) J. Biol. Chem. 256, 9284-9288
7. Howe, C. L., Mooseker, M. S., and Graves, T. A. (1980) J. Cell Biol. 88, 916-922
8. Glenney, J. R., Jr., Betescher, A., and Weber, K. (1980) Proc. Natl. Acad. Sci. U. S. A. 77, 6458-6462
9. Hesterberg, L. K., and Weber, K. (1982) J. Biol. Chem. 257, 359-364
10. Matsudaira, P. T., and Burgess, D. R. (1982) J. Cell Biol. 92, 648-656
11. Hummel, J. P., and Dreyer, W. J. (1962) Biochim. Biophys. Acta 63, 530-532
12. Glenney, J. R., Jr., Kauflus, P., and Weber, K. (1981) Cell 24, 471-480
13. Glenney, J. R., Jr., Geisler, N., Kauflus, P., and Weber, K. (1981) J. Biol. Chem. 256, 8156-8161
14. Glenney, J. R., Jr., and Weber, K. (1981) Proc. Natl. Acad. Sci. U. S. A. 78, 2810-2814
15. Argos, P. (1977) Biochemistry 16, 665-672
16. Chen, Y., Yong, I. T., and Martinez, A. M. (1972) Biochemistry 11, 4190-4131
17. Levi, A., Cimino, M., Meranti, D., and Calissano, P. (1974) Biochim. Biophys. Acta 365, 450-453
18. Hager, D. A., and Burgess, R. K. (1980) Anal. Biochem. 109, 76-86
19. Chou, P., and Fasman, G. D. (1974) Biochemistry 13, 211-222
20. Chou, P., and Fasman, G. D. (1974) Biochemistry 13, 222-245
21. Chou, P., and Fasman, G. D. (1977) Trends Biochem. Sci. 2, 128-131
22. Ackers, G. (1973) Methods Enzymol. 27, 441-445
23. Cann, J. R., and Hinman, N. D. (1976) Biochemistry 15, 4614-4622
24. Iole, T., and Okuyama, T. (1978) Eur. J. Biochem. 89, 379-388
25. Szczepanyi, D. M. E., Obendorf, S. K., and Muffat, K. (1981) Nature 294, 232-332
26. Mooews, P. C., and Kreisring, R. H. (1975) J. Mol. Biol. 91, 229-232
27. Kretsing, R. H., and Barry, C. D. (1975) Biochem. Biophys. Acta 405, 40-52
28. Tuft, R. M., and Kretsing, R. H. (1975) Science 187, 167-169