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# End-Label Fingerprintings Show That an N-Terminal Segment of Depactin Participates in Interaction with Actin†

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*Received June 8, 1988; Revised Manuscript Received July 27, 1988*

ABSTRACT: A 1:1 complex of actin and depactin, an actin-depolymerizing protein isolated from starfish oocytes [Mabuchi, I. (1983) *J. Cell Biol. 97*, 1612-1621], was cross-linked with 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC) to introduce covalent bonds at their contact site. Locations of cross-linking sites were identified along the depactin sequence by the end-label fingerprinting, which employed site-directed antibodies against the N- and C-termini of depactin as end labels. Mappings with these end labels have revealed that the N-terminal segment of depactin (residues 1-20) contains sites in contact with the N- and C-terminal segments of actin, both of which participate in interaction with depactin [Sutoh, K., & Mabuchi, I. (1986) *Biochemistry 25*, 6186-6192].

Actin is one of major components of cytoskeletal structures and motile apparatus in eukaryotic cells. Association and dissociation of actin and a large number of actin-binding proteins are essential for a dynamic change of cytoskeletons and motile apparatus in living cells. In order to understand the molecular mechanism underlying these events, it is important to elucidate the structural basis of interaction between actin and actin-binding proteins.

Since there are many types of actin-binding proteins in a cell while surface area on actin, especially on F-actin, is very limited, it is a reasonable speculation that some actin-binding proteins share their binding sites with others. In fact, it has been shown that the N- and C-terminal segments of actin participate in binding many actin-binding proteins: myosin (Sutoh, 1982a), depactin (Sutoh & Mabuchi, 1984, 1986), fragmin (Sutoh & Hatano, 1986), troponin I (Grabarek & Gergely, 1987), $\alpha$-actinin, actinogelin (Mimura & Asano, 1987), gelsolin (K. Sutoh and H. L. Yin, unpublished results), and profilin (T. Pollard, D. Kaiser, C. Ampe, and J. Vandekerckhove, personal communication). In spite of the wide

† This work was supported by grants-in-aid from the Ministry of Education, Science and Culture of Japan and a basic research grant from the Muscular Dystrophy Association.

0006-2960/89/0428-0102$01.50/0 

variety of effects of these proteins on actin, they share the N- and C-terminal segments of actin as part of their contact sites. Thus, an interesting question is if there is a common sequence in actin-binding proteins to recognize these segments of actin. A recent finding that profilin, a small actin-depolymerizing protein, has a sequence homologous to that of gelsolin (Ampe & Vandekerckhove, 1987) might suggest the presence of such a common sequence.

Depactin ($M_r$ 17000) is an actin-depolymerizing protein isolated from starfish oocytes (Mabuchi, 1981). It binds to F-actin and sequesters actin monomer to form a 1:1 actin–depactin complex (Mabuchi, 1982, 1983). The amino acid sequence of depactin (Takagi et al., 1988) does not show any homology to those of other actin-binding proteins published so far, indicating that this is a unique member of the actin-binding proteins.

Our previous work has shown that this protein interacts with both the N- and C-terminal segments of actin (Sutoh & Mabuchi, 1984, 1986). In this paper, by employing the end-label fingerprinting, we identified a depactin segment that participates in interaction with these actin segments.

## Materials and Methods

*Proteins and Reagents.* Depactin was prepared as previously described (Mabuchi, 1983). Actin was prepared according to the method of Spudich and Watt (1971). $^{35}$S-Labeled protein A was purchased from Amersham (Amersham, England). Lysyl endopeptidase (LEP)[1] was from Wako Chemical Co. (Osaka, Japan). Keyhole limpet hemocyanin (KLH) was purchased from Calbiochem-Behring (La Jolla, CA). Cyanogen bromide (CNBr) and 1-ethyl-3-[3-(dimethylamino)-propyl]carbodiimide (EDC) were from Wako Chemical Co.

*Site-Directed Antibodies.* Peptides with the following sequences were synthesized by the solid-phase method and purified by HPLC: (1) Pro-Gln-Ser-Gly-Thr-Ala-Leu-Asp-Glu-Asn-Val-Lys-Glu-Glu-Ile-Cys and (2) Cys-Ser-Glu-Glu-Ala-Ile-Gly-Asp-Lys-Ile-Lys-Asn-Phe. The former corresponds to the N-terminal sequence of depactin and the latter to its C-terminal sequence (Takagi et al., 1988) except for a cysteine residue, which was used as an anchoring site to a carrier protein (KLH). These peptides were coupled with KLH as described (Sutoh et al., 1987) The resulting peptide–KLH conjugate was injected into rabbits (1 mg of conjugate per rabbit) with complete Freund's adjuvant. After 2 and 4 weeks, these rabbits had additional injections of 1 mg of conjugate with complete Freund's adjuvant. Antisera were collected 1 week after the final injection. Titers of antisera were monitored after each injection by Western blotting (Towbin et al., 1979).

*Cross-Linking of the Actin–Depactin Complex.* A 1:1 complex of actin and depactin formed in 0.1 M NaCl, 20 mM MOPS, and 2 mM $MgCl_2$ (pH 7.5) was cross-linked with 20 mM EDC for 2 h at 25 °C. The reaction was quenched by addition of $^1/_{50}$ volume of 2-mercaptoethanol and $^1/_5$ volume of 10% $NaDodSO_4$. The solution was immediately boiled for 5 min.

*CNBr Mappings of Cross-Linking Site.* After the cross-linking reaction, the reaction mixture was electrophoresed in a $NaDodSO_4$ slab gel [12.5% acrylamide–0.4% bis(acrylamide)] (Laemmli, 1970) in duplicate. The duplicate gel lanes were cut out and washed with 50% methanol for 30 min twice and then with methanol for 30 min. The resulting gel slices were dried in vacuo. To these dried gels was added 0.3 M CNBr in 70% formic acid. The cleavage reaction was allowed to proceed for 1.5 h at 37 °C and then quenched by washing the gels with 50% methanol containing 10% 2-mercaptoethanol. The gels were washed with 50% methanol for 30 min and then with methanol for 30 min. They were dried in vacuo. These duplicate gels were soaked in 2% $NaDodSO_4$, 10 mM Tris-HCl, 2% 2-mercaptoethanol, and 10% glycerol (pH 8.0) for 1 h at 37 °C and then directly loaded onto slab gels [15% acrylamide–0.5% bis(acrylamide)]. After electrophoresis, separated CNBr fragments were transferred onto Durapore membranes (Millipore) (Towbin et al., 1979) in duplicate. These membranes were soaked in 0.15 M NaCl, 20 mM Tris-HCl, and 0.05% Tween 20 (ELISA grade, Bio-Rad) (TTBS) for 15 min twice with gentle shaking. Then one of the membranes was soaked in TTBS containing the antiserum against the depactin N-terminal segment while the other was soaked in TTBS containing the antiserum against the depactin C-terminal segment. These antisera were diluted 2000-fold. After being gently shaken for 1 h, these membranes were washed with TTBS for 5 min three times. The membranes were then soaked in TTBS containing 2 μCi of $^{35}$S-labeled protein A (Amersham) for 1 h with gentle shaking. Membranes were then washed with TTBS for 5 min three times and dried. These membranes were layered on X-ray films (Kodak XAR-5) and kept at −80 °C for several days for autoradiography.

*LEP Mappings of Cross-Linking Sites.* The reaction mixture of EDC cross-linking was electrophoresed in a slab gel [12.5% acrylamide–0.4% bis(acrylamide)] in duplicate. After electrophoresis, duplicate gel lanes were cut out and loaded onto slab gels [15% acrylamide–0.5% bis(acrylamide)] whose stacking gels contain 1 μg/mL lysyl endopeptidase (LEP). Peptides were partially cleaved in the stacking gel during electrophoresis. Fragments generated by the LEP digestion were transferred onto membranes after electrophoresis. One of the duplicate membranes was stained with the anti-N-terminal antibody while the other was stained with the anti-C-terminal antibody as above.

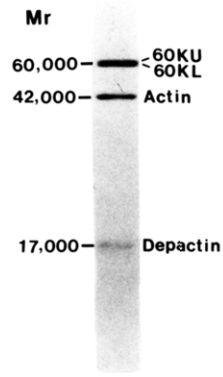


FIGURE 1: Cross-linking of actin and depactin. EDC cross-linking generates two cross-linked products (60KU and 60KL) with slightly different mobilities in the $NaDodSO_4$–acrylamide gel [12.5% acrylamide–0.4% bis(acrylamide)]. Cross-linking conditions: actin, 0.5 mg/mL; depactin, 0.25 mg/mL; solvent, 0.1 M NaCl, 20 mM MOPS, and 2 mM $MgCl_2$ (pH 7.5); cross-linker, 20 mM EDC; temperature, 25 °C; reaction time, 2 h.

## Results

*Cross-Linking of Actin and Depactin with EDC.* A 1:1 complex of actin and depactin was cross-linked with a zero-length cross-linker EDC as described (Sutoh & Mabuchi, 1984, 1986). As shown in Figure 1, the EDC reaction gen-

[1] Abbreviations: Tris, tris(hydroxymethyl)aminomethane; MOPS, 3-(*N*-morpholine)propanesulfonic acid; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; CNBr, cyanogen bromide; $NaDodSO_4$, sodium dodecyl sulfate; LEP, lysyl endopeptidase.

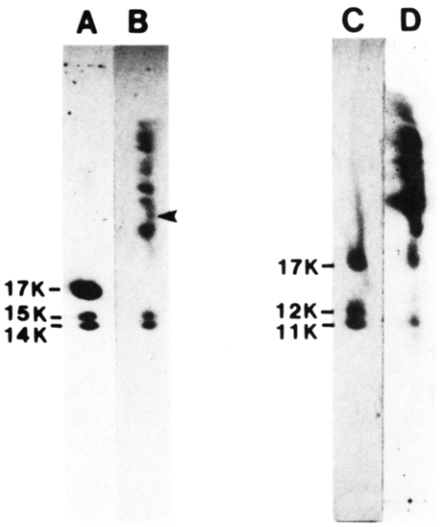


FIGURE 2: CNBr mapping of cross-linking sites of the N- and C-terminal segments of actin along the depactin sequence. CNBr fragments were stained with the antibody against the C-terminal segment of depactin (lanes A and B) or with the antibody against its N-terminal segment (lanes C and D). Lanes A and C: CNBr fragments generated from non-cross-linked depactin. Lanes B and D: CNBr fragments generated from the 60KU and 60KL products. For lane D, X-ray film was exposed for a prolonged period of time to make it sure that the 11K and 12K fragments were not released from the 60KU and 60KL products.

erated two cross-linked products (apparent $M_r$ ~60000) with slightly different mobilities in a $NaDodSO_4$–acrylamide gel (the 60KU and 60KL cross-linked products). The difference in mobilities was apparent only after prolonged electrophoresis, consistent with our previous observation (Sutoh & Mabuchi, 1986). We have already shown that one of the products with lower mobility (the 60KU product) is an actin–depactin conjugate in which depactin is cross-linked to the N-terminal segment of actin while the other product with higher mobility (the 60KL product) is an actin–depactin conjugate in which depactin is cross-linked to the C-terminal segment of actin (Sutoh & Mabuchi, 1986).

*CNBr Mappings of Cross-Linking Sites of Actin along the Depactin Sequences.* The strategy for locating cross-linking sites of actin along the depactin sequence is similar to that described previously (Sutoh & Mabuchi, 1986). Non-cross-linked depactin and the two actin–depactin conjugates, i.e., the 60KU and 60KL products, were separated in a $NaDodSO_4$ gel (the first-dimension gel) in duplicate, though the 60KU and 60KL bands were very closely spaced. They were then partially digested by CNBr in gel strips. After the partial CNBr cleavage, these duplicate gel strips were directly loaded onto two slab gels (the second-dimension gel) to separate CNBr fragments generated from each peptide. After these fragments were transferred onto the membranes, one of the duplicate membranes was stained with the antibody against the depactin C-terminus while the other was stained with the antibody against the depactin N-terminus.

The membrane stained with the anti-C-terminal antibody showed that two fragments with apparent molecular weights of 14000 and 15000 were generated from the non-cross-linked depactin upon CNBr cleavage (lane A in Figure 2). Together with the 14K and 15K bands, faint bands were also detected in the lower molecular weight region when X-ray films were exposed for prolonged period of time (data not shown). CNBr fragments visible on the membrane necessarily contained the C-terminus of depactin since they were stained with the antibody against the region. Examination of the depactin sequence (total 150 residues) (Takagi et al., 1988) has revealed that there are four methionine residues in the sequence, i.e., Met-20, Met-30, Met-101, and Met-110. Judging from their apparent molecular weights, the 15K and 14K fragments were assigned as depactin fragments of residues 21–150 and residues 31–150, respectively. Smaller C-terminal fragments of residues 102–150 and 111–150 were faintly stained on the membrane possibly because they were only partially trapped on it due to their small size.

The 60KU and 60KL actin–depactin conjugates released the 14K and 15K fragments together with higher molecular weight fragments upon CNBr digestion (lane B in Figure 2). Intensity ratios of the 14K and 15K bands to other bands were very similar in lanes A and B. These results indicate that the actin–depactin conjugates released free depactin fragments of residues 21–150 (15K) and residues 31–150 (14K) upon CNBr cleavage. Therefore cross-linking sites of both the N- and C-terminal segments of actin must be outside the depactin segment of residues 21–150, i.e., within its N-terminal segment of residues 1–20 (Figure 4).

The membrane stained with the anti-N-terminal antibody showed that non-cross-linked depactin released two N-terminal fragments with $M_r$ 11000 and 12000 (lane C in Figure 2). From the depactin sequence, the 11K and 12K fragments were assigned as residues 1–101 and 1–110, respectively. Smaller N-terminal fragments were only faintly stained on the membrane. The 60KU and 60KL products did not release the 12K N-terminal fragments of depactin upon the CNBr digestion. They released only a small amount of the 11K fragment (lane D in Figure 2). Almost all the fragments stayed in the higher molecular weight region on the blot. The results indicate that cross-linking sites of both the N- and C-terminal segments of actin are within the N-terminal depactin segment of residues 1–101 (Figure 4).

*LEP Mappings of Cross-Linking Sites of Actin along the Depactin Sequence.* Non-cross-linked depactin and the two actin–depactin conjugates were separated in the first-dimension gel and then loaded on the second-dimension gel. These peptides were partially digested with lysyl endopeptidase (LEP) during electrophoresis (Materials and Methods). After electrophoresis, LEP fragments were transferred onto membranes in duplicate. One of the duplicate membranes was stained with the antibody against the depactin C-terminus and the other was stained with the antibody against the depactin N-terminus as above (Figure 3).

The membrane stained with the anti-C-terminal antibody showed that non-cross-linked depactin released a series of C-terminal fragments (lane A in Figure 3). A similar fragment pattern was generated from the 60KU and 60KL actin–depactin conjugates (lane B in Figure 3). Both patterns were aligned with each other up to the 12K fragment, which was assigned as residues 45–150, 48–150, and/or 49–150 judging from its apparent molecular weight and the depactin sequence (Takagi et al., 1988). Thus, cross-linking sites of both the N- and C-terminal segments of actin are located outside the C-terminal 12K fragment of depactin which spans residues 45–150, 48–150, and/or 49–150, i.e., within residues 1–49 (Figure 4).

When the membrane was stained with the anti-N-terminal antibody, the non-cross-linked depactin and the actin–depactin conjugates generated very different fragment patterns (lanes C and D in Figure 3). None of the fragments released from the 60KU and 60KL products were aligned with those released from non-cross-linked depactin. The smallest depactin fragment released from non-cross-linked depactin had a $M_r$ of

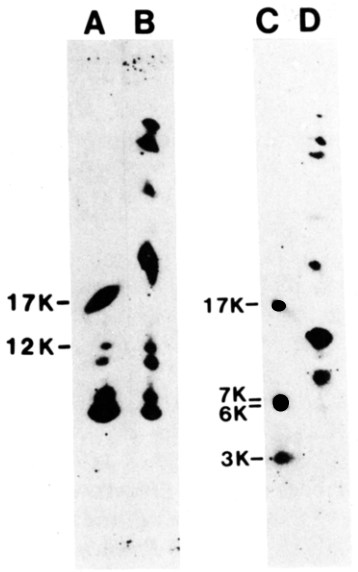


FIGURE 3: LEP mapping of cross-linking sites of the N- and C-terminal segments of actin along the depactin sequence. LEP fragments were stained with the antibody against the C-terminal segment of depactin (lanes A and B) or with the antibody against its N-terminal segment (lanes C and D). Lanes A and C: LEP fragements generated from non-cross-linked depactin. Lanes B and D: LEP fragments generated from the 60KU and 60KL products.

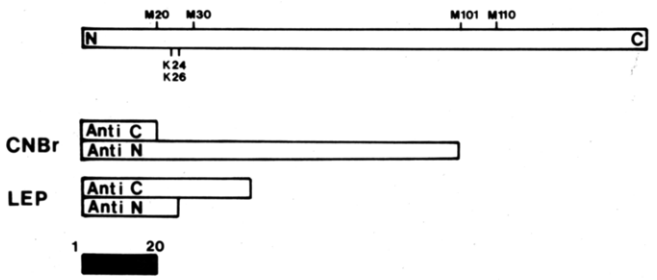


FIGURE 4: Identification of cross-linking sites of the N- and C-terminal segments of actin along the depactin sequence. Top bar represents the depactin sequence, on which residues mentioned in the text are indicated. Open bars in the lower part represent depactin segments identified as participating in interaction with the actin segments. CNBr: CNBr cleavages. LEP: LEP cleavages. Anti C: segments were identified by the use of antibody against the C-terminus of depactin. Anti N: segments were identified by the use of the antibody against the N-terminus of depactin. A segment shown by a closed bar is derived from these maps as the one containing the cross-linking sites.

3000. Considering the fact that the 3K fragment migrated slightly behind the CNBr fragment of residues 1–20 (data not shown), the 3K fragment was assigned as residues 1–24 and/or 1–26. Thus, it is concluded that cross-linking sites of both the N- and C-terminal segments of actin are located within residues 1–24 (Figure 4).

Figure 4 summarizes the CNBr and LEP maps of cross-linking sites of actin along the depactin sequence. From these maps, cross-linking sites of the N- and C-terminal segments of actin are located within a single N-terminal segment of depactin, which spans residues 1–20.

## Discussion

The N-terminal segment of depactin of residues 1–20 has been identified as cross-linking sites with the N- and C-terminal segments of actin (Figure 5). The N-terminal segment of actin has Asp-1, Glu-2, Asp-3, Glu-4, and Asp-11 as candidates for residues cross-linked with depactin (EDC cross-links amino and carboxyl groups when they are in contact) while the C-terminal segment has Lys-359, Glu-361, Asp-363,

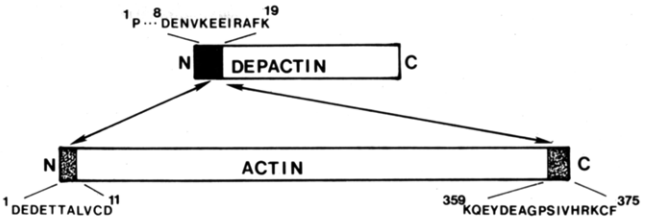


FIGURE 5: Interaction of actin and depactin. The top bar represents the depactin sequence. The lower bar represents the actin sequence. Sequences participating in the actin–depactin interaction are shown.

| | |
|---|---|
| Depactin (1-20) | PQSGTALDENVKEEIRAFKM |
| Profilin (95-122) | GVYNEKIQPGTAANVVEKLADYLIG |
| Myosin (641-660) | KGSSFQTVSALFRENLNKLM |

FIGURE 6: Sequences interacting with the N- and/or C-terminal segments of actin. Sequences of depactin (residues 1–20), profilin (residues 95–122), and myosin heavy chain (residues 641–660), which participate in interaction with the N- and/or C-terminal segments of actin, are shown. Potential cross-linking sites with the actin segments are underlined. The profilin sequence shown here is homologous to gelsolin and fragmin sequences (Ampe & Vandekerckhove, 1987). Residues numbers in the myosin sequence are from Maita et al. (1987).

Glu-364, Lys-373, and Phe-375 (Sutoh & Mabuchi, 1986). Examination of the depactin sequence (Takagi et al., 1988) indicates that Pro-1, Asp-8, Glu-9, Lys-12, Glu-13, Glu-14, and Lys-19 are candidates for residues cross-linked with those residues in the N- and C-terminal segments of actin.

It is very likely that these residues involved in the EDC cross-linking are in the contact site between actin and depactin, considering the following points. EDC cross-links closely juxtaposed amino and carboxyl groups in tightly complexed proteins. For example, actin and myosin are cross-linked with EDC only when they form a rigor complex. No cross-linking occurs when they dissociate in the presence of MgATP (Mornet et al., 1982). Actin and gelsolin C-terminal fragments are cross-linked only in the presence of calcium, which induces complex formation of actin and gelsolin (K. Sutoh and H. L. Yin, unpublished results). Tight association of proteins is a prerequisite for their cross-linking by EDC.

We now know the depactin sequence which participates in interaction with the N- and C-terminal segments of actin. An interesting question is whether similar sequences are found in other actin-binding proteins. Amino acid sequences of the following actin-binding proteins, all of which bind to the N- and/or C-terminal segments of actin, have been determined: profilin (Ampe et al., 1985), gelsolin (Kwiatkowski et al., 1986), fragmin (Ampe & Vandekerckhove, 1987), myosin (Gallagher & Elzinga, 1980; Maita et al., 1987), and troponin I (Wilkinson & Grand, 1978). No sequence homologous to the N-terminal segment of depactin (residues 1–20) has been detected in these proteins. In this context, it must be mentioned that Pollard, Kaiser, Ampe, and Vandekerckhove have shown that profilin is cross-linked with the C-terminal segment of actin at Lys-115 (personal communication). Furthermore, Ampe and Vandekerckhove (1987) have shown that the profilin sequence around Lys-115 is homologous to sequences in fragmin and gelsolin and have suggested that these homologous sequences participate in interaction with actin. The profilin sequence is, however, not homologous to the depactin sequence of residues 1–20 (Figure 6).

The N-terminal CNBr segment of the 20K fragment of myosin heavy chain is cross-linked to the N-terminal segment of actin with EDC (Sutoh, 1982b, 1983). The sequence contains two lysine residues (Figure 6) (Gallagher & Elzinga, 1973; Maita et al., 1987), which are candidates for a residue

cross-linked with the N-terminal segment of actin. The myosin sequence around these lysine residues is homologous neither to the depactin sequence of residues 1-20 nor to the profilin sequence around Lys-115 (Figure 6).

Thus no homology has been detected among sequences (depactin, profilin, and myosin) which participate in interaction with the N- and/or C-terminal segments of actin. These results are contrary to the idea that some actin-binding proteins have a common sequence which recognizes the N- and/or C-terminal segment of actin. It seems that a variety of sequences can recognize these actin segments.

ACKNOWLEDGMENTS

We thank Dr. T. Takagi for carrying out homology search of depactin and other actin-binding proteins.

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# Interaction of Tetrahydropteroylpolyglutamates with Two Enzymes from Mitochondria†

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*Received May 27, 1988; Revised Manuscript Received August 3, 1988*

ABSTRACT: The dissociation constants of tetrahydropteroylpolyglutamates, having from one to six glutamate residues, have been determined for the two mitochondrial enzymes serine hydroxymethyltransferase and dimethylglycine dehydrogenase. The ratios of the dissociation constants for the mono- and hexaglutamate forms of the coenzyme were 200 and less than 10 for serine hydroxymethyltransferase and dimethylglycine dehydrogenase, respectively. $K_m$ and $k_{cat}$ values were determined for the reversible interconversion of serine and glycine as a function of the number of glutamyl residues on the coenzyme. The values in the serine to glycine direction did not significantly change with the number of glutamyl residues, but in the glycine to serine direction, there was a 9-fold increase in the $k_{cat}/K_m$ when the longer chain polyglutamates were used as the coenzyme substrate. A sensitive and rapid method for determining the dissociation constants of proteins which bind either tetrahydropteroylpolyglutamates or their 5-methyl and 5-formyl conjugates is described.

The intracellular folate pool exists mainly as polyglutamates having from four to eight glutamyl residues linked through the γ-carboxyl group (Baugh & Krumdieck, 1971; Brown et al., 1974; Scott & Weir, 1976). The number of glutamyl residues varies with both species and tissue (Cossins, 1984; Krumdieck & Eto, 1986). The active forms of this coenzyme are the reduced compounds, which are referred to collectively as tetrahydropteroylpolyglutamates. Cook and Blair (1979) found that in rat liver nearly all the intracellular folate pool is localized in the cytosol and the mitochondria, with the coenzyme being distributed about equally between these two cellular compartments. Exchange between the two folate pools is not known to occur with the monoglutamyl derivative (Cybulski & Fisher, 1976).

Many of the enzymes which utilize this coenzyme have been catagorized with regard both to the specificity of these enzymes for a particular polyglutamate chain length and to the effect

† This work was supported by Grant GM 28143 from the National Institutes of Health.
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0006-2960/89/0428-0106$01.50/0 