Interaction of Vitamin K Dependent Proteins with Membranes†

Gary L. Nelsestuen,*,‡ Walter Kisiel,§ and Richard G. Di Scipio

ABSTRACT: The membrane-binding characteristics of six vitamin K dependent plasma proteins, which have homologous amino acid sequences, were compared. All of these proteins display calcium-dependent membrane binding and the identified equilibria for protein–membrane binding are qualitatively the same for all proteins. Quantitative characteristics of these protein–membrane interactions allow organization into distinct subgroups. Protein C and factor VII form a subgroup which has extremely low affinity for bilayer membranes; prothrombin, factor X, and protein S form the tightest complexes with membranes and factor IX displays intermediate affinity. In the presence of manganese (which substitutes for calcium in a cation-dependent protein transition), calcium titration of protein–membrane binding shows the same calcium dependence for all proteins except prothrombin which requires lower calcium. These protein–membrane binding characteristics agree very well with the relatedness of these proteins based on their partial amino-terminal sequences.

The known vitamin K dependent proteins of the plasma consist of the blood clotting proteins, prothrombin, factor X, factor IX, and factor VII along with two other proteins identified as protein C (Stenflo, 1976) and protein S (Di Scipio et al., 1977). The vitamin K dependent structure in these proteins, γ-carboxyglutamic acid, is involved in calcium-mediated protein–membrane interactions. Recent studies applying several techniques have provided considerable quantitative and qualitative information about the prothrombin- and factor X–membrane interactions. For example, the following equilibria are consistent with the observed properties of prothrombin- and factor X–membrane binding (Nelsestuen & Lim, 1977):

$$\left.\begin{array}{l} P + i\mathrm{Ca} \overset{1}{\rightleftharpoons} P_{i\mathrm{Ca}} \overset{2}{\rightleftharpoons} P'i\mathrm{Ca} \\ \qquad\qquad\qquad\quad + \\ PL + j\mathrm{Ca} \overset{3}{\rightleftharpoons} PL_{j\mathrm{Ca}} \end{array}\right\} + m\mathrm{Ca} \overset{4}{\rightleftharpoons} P'\cdot PL_{i+j+m\mathrm{Ca}} \quad (1)$$

P is protein, P′ is protein after undergoing a calcium-dependent protein transition, PL is a phospholipid membrane, and P′·PL is the protein–membrane complex. The values of i, j, and m are interdependent and m becomes zero when i and j are saturated.

For measurement of the amount of membrane-bound protein under any set of conditions, a dissociation constant can be determined which, according to the equilibria above, is identified as (Nelsestuen & Lim, 1977):

$$K_4^{\mathrm{Ca}*} = \frac{[P_{i\mathrm{Ca}} + P'_{i\mathrm{Ca}}][PL_{j\mathrm{Ca}}]}{[P'\cdot PL_{i+j+m\mathrm{Ca}}]} \quad (2)$$

The value of $K_4^{\mathrm{Ca}*}$ is the dissociation constant for protein–membrane binding at a constant calcium concentration and extensive experiments with prothrombin and factor X have been reported (Nelsestuen & Broderius, 1977). The results revealed that, quantitatively, the various reactions in eq 1 and 2 differ considerably for these two proteins.

The present investigations were undertaken to compare the remaining vitamin K dependent proteins to determine if they all display membrane binding, to what extent they are similar to prothrombin and factor X and to determine possible relative homologies within this class of proteins.

Materials and Methods

All proteins were purified to homogeneity by published procedures and were quantitated by their extinction coefficients at 280 nm. The proteins and their extinction coefficients ($\epsilon_{280\mathrm{nm}}^{1\%}$) are as follows: bovine prothrombin (Nelsestuen & Suttie, 1972), 14.1 (Cox & Hanahan, 1970); bovine factors X_1 and X_2, 12.4 (Fujikawa et al., 1972); bovine factor IX, 14.9, and factor IXa, 14.3 (Fujikawa et al., 1974); bovine protein C, 13.7 (Kisiel et al., 1976); bovine factor VII, 12.9 (Kisiel & Davie, 1975; Radcliff & Nemerson, 1976); and human protein S, estimated to be 10.0 (Di Scipio et al., 1977).

Phospholipids were purchased from the Sigma Chemical Co. and were >95% pure (suppliers estimates). Single bilayer vesicles were prepared as described previously (Nelsestuen & Lim, 1977). Phospholipid concentrations were determined from measurement of organic phosphorus (Chen et al., 1956) utilizing a weight conversion factor of 25 (phosphorus/phospholipid) (Nelsestuen & Lim, 1977). DNP-PE[1] was synthesized from 1,2-dipalmitoyl-*sn*-glycerol-3-phosphorylethanolamine and 2,4-dinitro-1-fluorobenzene as described previously (Nelsestuen et al., 1976).

Protein–membrane binding was measured by either the fluorescence energy transfer technique or relative light scattering. For fluorescence energy transfer the phospholipid consisted of 60% DNP-PE–40% phosphatidylserine and protein–membrane binding was measured by fluorescence quenching (Nelsestuen et al., 1976). Basically, the phospholipid, protein, and calcium are mixed in the cuvette and the fluorescence (f) is measured; EDTA is added to remove calcium from the complex thereby releasing the protein from the membrane and the fluorescence (f_0) is again measured. The

† From the Department of Biochemistry, University of Washington, Seattle, Washington 98195. *Received November 16, 1977.* Supported in part by Grants HL-16919 and HL-15728 from the National Institutes of Health.

‡ An Established Investigator of the American Heart Association on temporary leave of absence from the University of Minnesota. Current address: Department of Biochemistry, College of Biological Sciences, University of Minnesota, St. Paul, Minn. 55108.

§ An Established Investigator of the American Heart Association.

[1] Abbreviations used: DNP-PE, 1,2-dipalmitoyl-*sn*-glycerol 3-(*n*-2,4-dinitrophenylaminoethyl)phosphate; EDTA, ethylenediaminetetraacetic acid.

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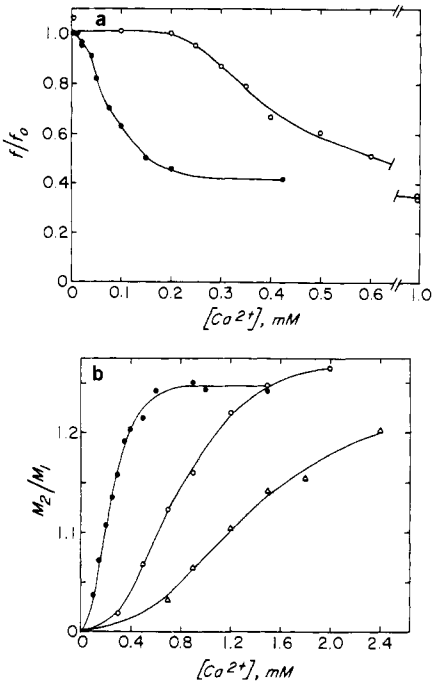

FIGURE 1: Measurement of protein C-membrane binding. Figure 1a shows the calcium titration of protein C (40 μg/mL) binding to the membranes (200 μg/mL) used in fluorescence energy transfer. Titrations in the presence (●) and absence (○) of 0.2 mM manganous ion are given. Figure 1b shows calcium titration of protein C (370 μg/mL) binding to membranes (26 μg/mL) of 20% phosphatidylserine measured by the relative light scattering technique. Titration of protein C in the presence (●) and absence (○) of 0.2 mM manganous ion and of activated protein C in the absence of manganous ion is also shown (△).

value of f/f_0 is then plotted vs. calcium concentration. The protein/phospholipid ratios used were 1:5 (w/w).

Protein–membrane binding was measured by relative light scattering intensities using a membrane of 20% phosphatidylserine–80% phosphatidylcholine by the technique described previously (Nelsestuen & Lim, 1977). The relative 90° light scattering intensity was determined for phospholipid vesicles (I_{s_1}) in a Perkin-Elmer Model 44A fluorescence spectrofluorometer (excitation and emission monochrometers were set at 350 nm). Calcium and/or protein were added and the relative light scattering intensity (I_{s_2}) was measured. The values were corrected for light scattering by unbound protein and the relative molecular weight of the protein–membrane complex was determined from the relationship:

$$\frac{I_{s_2}}{I_{s_1}} = \left(\frac{\partial n_2/\partial c_2}{\partial n_1/\partial c_1}\right)^2 \left(\frac{M_2}{M_1}\right)^2 \qquad (3)$$

where M_1 is the molecular weight of the phospholipid vesicles, M_2 is the molecular weight of the protein–vesicle complex, and $\partial n/\partial c$ is the refractive index increment for each of these species. In these calculations the value of $\partial n_2/\partial c_2$ was estimated from a value for protein (0.192) and phospholipid (0.172) (Nelsestuen & Lim, 1977) and the quantity of each of these components in the complex. The value obtained is a statistical average and the method of calculation was given previously. All proteins were assumed to have a $\partial n/\partial c$ value of 0.192, which is the value determined for prothrombin (Nelsestuen & Lim, 1977). The narrow range of $\partial n/\partial c$ reported for a variety of proteins (Doty & Edsall, 1951) indicates that this assumption is valid for the data interpretation presented here. Application of eq 3 to the relative light scattering measurements allows calculation of the free and bound protein concentrations when a known amount of protein is added to a known amount of phospholipid. Dissociation constants for protein–membrane binding (K_4^{Ca*}, eq 2) can then be estimated as described previously (Nelsestuen & Lim, 1977).

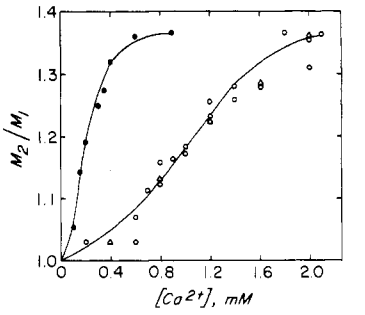

FIGURE 2: Factor IX–membrane binding. The calcium titrations of factor IX (135 μg/mL) binding to membranes of 20% phosphatidylserine (20 μg/mL) in the presence (●) and absence (○) of 0.2 mM manganous ion are shown. A titration following activation of factor IX with Russell's viper venom (Lindquist et al., 1976) is also shown (△).

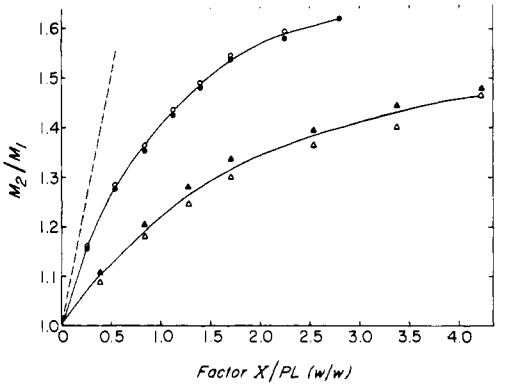

FIGURE 3: Factor X_1- and X_2-membrane binding. The technique of relative light scattering was used to measure the relative molecular weight of the protein–membrane complex as outlined in Methods. The membrane vesicles are assigned a molecular weight of 1.0 and the dotted line shows the theoretical molecular weight of the complex if all of the added factor X were bound to the membrane. The curves shown are for factor X_1 in 1 mM calcium (▲), factor X_2 in 1 mM calcium (△), factor X_1 in 2 mM calcium (●), and factor X_2 in 2 mM calcium (○).

All studies were carried out at 25 °C in a buffer solution of 0.05 M Tris (pH 7.5)–0.1 M NaCl. Reversibility of protein–membrane binding was always tested by the addition of EDTA to dissolve the complex.

Results

Figures 1–3 give some representative data obtained by the methods employed and Table I summarizes the pertinent characteristics of these protein–membrane interactions. The calcium titration curves obtained were invariably sigmoidal (e.g., Figures 1 and 2).

The phospholipid used in measurement of protein–membrane binding by fluorescence energy transfer gave maximum quenching of about 60% (Figure 1a) for all proteins studied. Factor IX–membrane binding could not be studied by this technique due to a rapid (considerable binding occurring in 10 to 60 s) calcium-independent protein–membrane interaction which is also not inhibited by EDTA (indeed, EDTA appeared to stimulate this interaction). While calcium-independent binding to this very acidic membrane was observed for several of the proteins, it is usually slow and does not preclude the judicious use of this technique. The rates of the calcium-independent binding for the other proteins required times of minutes to hours depending on the protein. Except for prothrom-

TABLE I: Vitamin K Dependent Protein-Membrane Binding.

Protein	Fluoresence energy transfer[a]		Relative light scattering[b]		
	$Ca_{0.5}$[c] (+Mn)	$Ca_{0.5}$ (−Mn)	$Ca_{0.5}$[c] (+Mn)	$Ca_{0.5}$ (−Mn)	K_4^{Ca*} (μM)
Prothrombin	0.032	0.2	0.08	0.03	0.6 M
Factor X	0.09	0.45	0.18	0.5	0.25
Factor IX			0.20	1.0	2
Factor VII	0.075	0.65	0.18	0.47	15
Protein C	0.08	0.4	0.22	0.75	17
Protein S	0.09	0.25	0.17	0.5	<1

[a] The phospholipid used is 60% DNP-PE–40% phosphatidylserine and typical results are shown in Figure 1a. [b] The phospholipid used is 20% phosphatidylserine–80% phosphatidylcholine and typical results are shown in Figures 1b and 2. K_4^{Ca*} is the dissociation constant for protein–membrane binding at a constant calcium concentration (2 mM in this case). The identity of K_4^{Ca*} and its method of determination are as given previously (Nelsestuen & Lim, 1977). [c] $Ca_{0.5}$ is the calcium concentration (mM) at the midpoint of the cooperative protein-membrane binding curve.

bin, where a slow protein transition retards the rate, the calcium-dependent membrane binding was complete in less than 10 s, was reversible and therefore could be easily distinguished from calcium-independent binding. The method employed (Nelsestuen et al., 1976) assures measurement of the reversible calcium-dependent interaction only.

The fluorescence quenching resulting from the irreversible calcium-independent membrane binding is nearly quantitative indicating that all areas of the protein are closely associated with the membrane. The calcium-dependent membrane binding involves no detected penetration of the protein into the lipid area of the membrane (Lim et al., 1977) and shows only a 60% quenching of fluorescence (Figure 1a). Calcium-independent binding of other vitamin K dependent proteins to very acidic membranes has been previously reported for prothrombin (Bull et al., 1972) and protein C (Esmon et al., 1976). The bound prothrombin had a low clotting activity, perhaps indicating that it was not available on the membrane surface. This calcium-independent membrane binding does not appear to be related to γ-carboxyglutamic acid since prothrombin intermediate I, which contains none of these residues, also displays calcium-independent membrane binding to the very acidic membranes (unpublished observations). Calcium-independent membrane binding was not observed for membranes of physiological composition (i.e., $\leq$20% acidic phospholipid). Calcium-independent binding therefore appears to be an in vitro artifact of the use of very acidic membranes and is not of physiological significance. This interaction probably explains the inhibitory affect of very acidic membranes on the coagulation reactions (Bull et al., 1972); calcium-independent binding of factor Xa would remove this component from the prothrombinase complex and reduced thrombin generation would result. Of the proteins studied, prothrombin was the least susceptible and factors IX and Xa were the most susceptible to calcium-independent membrane binding.

Factors X_1 and X_2 have been reported to contain substantially different quantities of γ-carboxyglutamic acid (Neal et al., 1976). Therefore, the membrane-binding characteristics of factors X_1 and X_2 were compared. The results in Figure 3 demonstrate no difference (<3% for any determination) in the binding of factor X_1 and X_2 to membrane surfaces and it can be concluded that the membrane-binding site does not contribute to the difference between these proteins.

The summary of data (Table I) obtained shows several membrane-binding characteristics which are common to all of these vitamin K dependent proteins. First of all, while manganous ion is unable to effect protein-membrane binding by itself, its presence reduces the calcium concentration needed to bring about this interaction. This is explained by the ability of manganese to bring about the protein transition (reaction 2, eq 1) and thereby assist calcium in protein-membrane binding. Secondly, in the presence of manganese, calcium titration of protein-membrane binding is highly dependent on the membrane composition with more acidic membranes requiring lower calcium concentrations (e.g., compare Figures 1a and 1b or results in Table I for the two different membranes). This characteristic is a function of a minimum reaction 3 (eq 1): in the presence of manganese, calcium titration of protein-membrane binding is proposed to measure formation of a binding site nucleus on the membrane (Nelsestuen & Lim, 1977). This consists of acidic phospholipids and a minimum number of membrane-associated calcium ions (j in eq 1). More acidic membranes would have a higher affinity for calcium and would require lower calcium concentrations. This is the observed relationship (Table I). These characteristics agree with the more extensive studies on prothrombin and factor X (Nelsestuen & Broderius, 1977) and indicate that the model presented in eq 1 is characteristic of all of these vitamin K dependent proteins.

Another feature common to all proteins tested is that activation has only small (e.g., protein C, Figure 1b) or negligible (e.g., factor IX, Figure 2) effect on the membrane-binding affinity. Similar observations for prothrombin and factor X activation were reported previously (Nelsestuen et al., 1976; Nelsestuen & Lim, 1977). The significant change observed for protein C could be of biological importance, however, and should not be entirely dismissed.

With respect to other membrane-binding characteristics the vitamin K dependent proteins can be divided into distinct types or subgroups. First of all, the midpoint of the calcium titration curve in the presence of manganese is similar for all proteins (for the same phospholipid composition) except prothrombin. The model for protein-membrane binding (eq 1) would require that the minimum value for j (eq 1) in the case of prothrombin is lower than that for the other vitamin K dependent proteins and that the other vitamin K dependent proteins have approximately the same minimum value for j.

In the absence of manganese the calcium concentrations required for protein-membrane binding show considerable variation (Table I). This titration is dependent on both reactions 2 and 3 of eq 1 and it is concluded that the calcium concentration required for reaction 2 shows variation but the data do not allow its accurate estimation or grouping of these proteins on this basis.

Finally, we can observe that the dissociation constants for protein-membrane binding (K_4^{Ca*}) are quite variable. The order of membrane binding affinity is factor X > prothrombin > factor IX > factor VII $\geq$ protein C. The most significant correlation appears to be the relationship of protein C and factor VII which both have very low membrane-binding affinities. This low affinity undoubtedly explains the failure to observe calcium-dependent coelution of protein C and phospholipid during gel filtration chromatography (Esmon et al., 1976). An accurate value of K_4^{Ca*} for protein S was not de-

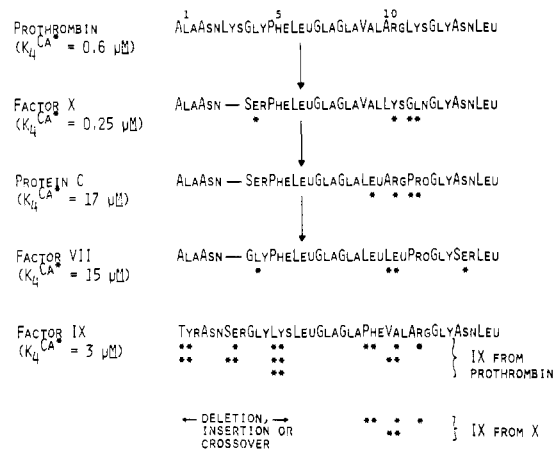

FIGURE 4: The evolutionary relationship of vitamin K dependent proteins. The amino-terminal sequence is given and the minimum number of mutations necessary for substitution of the particular amino acids are indicated by the asterisks directly below the amino acids. A single asterisk indicates a purine→purine or pyrimidine→pyrimidine type of mutation and a double asterisk indicates a purine↔pyrimidine type of mutation. The scheme presented represents the smallest number of mutations which could produce the sequences shown. K_4^{Ca*} represents the dissociation constant for protein binding to membrane vesicles of 20% phosphatidylserine at 2 mM calcium (from Table I). The proteins used are all from the bovine species and the sequences have been previously published (see Di Scipio et al., 1977).

termined due to the absence of an accurate extinction coefficient for this protein. The estimate obtained, however, suggests that it is more closely related to the proteins demonstrating higher membrane affinities.

Discussion

This comparison of vitamin K dependent proteins has demonstrated certain invariant characteristics of their membrane interactions and has also revealed differences which can be used to categorize these proteins into subgroups. The vitamin K dependent proteins of the plasma have homologous sequences and are undoubtedly descendent from a common ancestral gene (e.g., see Di Scipio et al., 1977). An interesting way of summarizing the results is in the form of an evolutionary pathway for appearance of these proteins (Figure 4) based on their degree of relatedness.

Figure 4 gives the amino-terminal sequences of five vitamin K dependent plasma proteins. The arrows indicate the probable evolutionary relationships of these proteins and the sequence of events shown represents the minimum number of mutational events required to produce the observed amino acid sequences. While this analysis includes only 13 or 14 amino acids, this relationship is corroborated by the membrane-binding characteristics of these proteins. First of all, prothrombin is unique with respect to its minimum value for j. Prothrombin should therefore occupy a terminal position in the evolutionary scheme. Mutations producing the first derivative of prothrombin changed the minimum value of j and the new characteristic was retained by all subsequent proteins. Prothrombin is also unique in that its membrane-binding site is removed from its enzymatic portion during the activation process. Prothrombin contains 10 γ-carboxyglutamic acid residues (Morris et al., 1976; Fernlund et al., 1975; Zytokovicz & Nelsestuen, 1975); factor X contains 12 (Enfield et al., 1975; Howard & Nelsestuen, 1975). Whether these latter observations are linked to the observed change in the minimum value of j will require further investigation.

A second feature of membrane-binding which correlates with the evolutionary sequence in Figure 4 is the relatedness of protein C and factor VII. These proteins have K_4^{Ca*} values which are two to three orders of magnitude higher than their plasma concentrations. This is not necessarily an anomaly since factor VII function involves phospholipids in the form of the lipoprotein, tissue factor (Bjorklid & Storm, 1977), rather than in the form of a bilayer membrane. It is possible that the loss of affinity for a bilayer membrane was accompanied by an increased affinity for the specific phospholipid arrangement provided by this lipoprotein. If this were the case, the function or activation of protein C may also involve a lipoprotein. Activated protein C is a potent inhibitor of blood coagulation but the details of its formation or action are not known (Kisiel et al., 1977). An interesting observation is that loss of membrane-binding affinity corresponds with the appearance of a proline residue at position 10 of these proteins. The change of secondary structure brought about by this substitution may be the actual cause of the altered membrane-binding affinity. Much further work will be required to test this interesting possibility.

Factor IX is unusual with respect to its amino-terminal five residues. It contains the same number of amino acids as prothrombin and analysis on this basis alone might suggest that it evolved directly from prothrombin. This possibility is unlikely since the number of point mutations needed to produce the five amino-terminal residues of factor IX from prothrombin is excessive (Figure 4) and factor IX is much more closely related to factor X with respect to its activation characteristics (see Davie & Fujikawa, 1975) and its minimum value for j (eq 1). Therefore, it seems most likely that factor IX evolved from factor X and that the amino-terminal five residues are the result of a deletion, insertion, or crossover mutation which retained the structural characteristics required by the vitamin K dependent proteins.

Protein S is not included in Figure 4 and only the human protein has been studied to date. The known amino-terminal sequence, however (Ala-Asn-Ser- ? -Leu), shows the deletion characteristic of factors X and VII and protein C. This protein is similar to factors X, IX, and VII and protein C with respect to its calcium titration in the presence of manganese (i.e., interpreted in this model to be the minimum value for j, eq 1). Additionally, this protein binds to membranes with an affinity characteristic of prothrombin or factor X. On these bases, protein S is more closely related to factor X than any other vitamin K dependent protein. More extensive work will be necessary to determine whether it is an intermediate or a branch protein in the scheme given in Figure 4.

Finally, a comforting observation is that the sequence of events provided in Figure 4 is supported by the logical evolution of modern blood coagulation. The most primitive system probably consisted of a single protein (prothrombin) and a simple activation process. Factor X would be the first protein to be derived and provided a more complex activation system with certain advantages. Factors IX and VII would then arise to provide more sophisticated methods of factor X activation. Protein C emerges as an evolutionary intermediate and this analysis will hopefully prove beneficial in determining the details of its function.

Another vitamin K dependent protein is found in bone (Price et al., 1976; Hauschka et al., 1975). This protein binds calcium very poorly (Price et al., 1977) but no studies on its membrane-binding ability have appeared.

Acknowledgments

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References

Bjorklid, E., & Storm, E. (1977) *Biochem. J. 165,* 89.
Bull, R. K., Jevons, S., & Barton, P. G. (1972) *J. Biol. Chem. 247,* 2747.
Chen, P. S., Toribara, T. Y., & Warner, H. (1956) *Anal. Chem. 28,* 1756.
Cox, A. C., & Hanahan, D. J. (1970) *Biochim. Biophys. Acta 207,* 49.
Davie, E. W., & Fujikawa, K. (1975) *Annu. Rev. Biochem. 44,* 799.
Di Scipio, R. G., Hermodson, M. A., Yates, S. G., & Davie, E. W. (1977) *Biochemistry 16,* 698.
Doty, P., & Edsall, J. T. (1951) *Adv. Protein Chem. 6,* 35.
Enfield, D. L., Ericsson, L. H., Walsh, K. A., Neurath, H., & Titani, K. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72,* 16.
Esmon, C. T., Stenflo, J., Suttie, J. W., & Jackson, C. M. (1976) *J. Biol. Chem. 251,* 3052.
Fernlund, P., Stenflo, J., Roepstorff, P., & Thomsen, J. (1975) *J. Biol. Chem. 250,* 6125.
Fujikawa, K., Legaz, M., & Davie, E. (1972) *Biochemistry 11,* 4882.
Fujikawa, K., Legaz, M. E., Kato, H., & Davie, E. W. (1974) *Biochemistry 13,* 4508.
Hauschka, P. V., Lian, J. B., & Gallop, P. M. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72,* 3925.
Howard, J. B., & Nelsestuen, G. L. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72,* 1281.
Huang, C. (1969) *Biochemistry 8,* 344.
Kisiel, W., & Davie, E. W. (1975) *Biochemistry 14,* 4928.
Kisiel, W., Ericsson, L. H., & Davie, E. W. (1976), *Biochemistry 15,* 4893.
Kisiel, W., Canfield, W. H., Ericsson, L. H., & Davie, E. W. (1977) *Biochemistry 16,* 5824.
Lim, T. K., Bloomfield, V. A., & Nelsestuen, G. L. (1977) *Biochemistry 16,* 4177.
Lindquist, P. A., Fujikawa, K., & Davie, E. W. (1976) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 35,* 1353 (abst).
Morris, H. R., Dell, A., Petersen, T. E., Sottrup-Jensen, L., & Magnusson, S. (1976) *Biochem. J. 153,* 663.
Mukhacheva, I. A., & Byshevskii, A. S. (1976) *Biokhimiya 41,* 1726.
Neal, G. G., Prowse, C. V., & Esnouf, M. P. (1976) *FEBS Lett. 66,* 257.
Nelsestuen, G. L. (1976) *J. Biol. Chem. 251,* 5648.
Nelsestuen, G. L., & Broderius, M. (1977) *Biochemistry 16,* 4172.
Nelsestuen, G. L., & Lim, T. K. (1977) *Biochemistry 16,* 4164.
Nelsestuen, G. L., & Suttie, J. W. (1972) *J. Biol. Chem. 247,* 6096.
Nelsestuen, G. L., Broderius, M., & Martin, G. (1976) *J. Biol. Chem. 251,* 6886.
Price, P. A., Otsuka, A. S., Poser, J. W., Kristaponis, J., & Raman, N. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73,* 1447.
Price, P. A., Otsuka, A. S., & Poser, J. W. (1977) in *Calcium Binding Proteins and Calcium Function* (Wasserman, R. H., et al., Eds.) p 333, Elsevier North Holland, Inc., New York, N.Y.
Radcliff, R., & Nemerson, Y. (1976) *J. Biol. Chem. 251,* 4797.
Stenflo, J. (1976) *J. Biol. Chem. 251,* 355.
Zytkovicz, T. H., & Nelsestuen, G. L. (1975) *J. Biol. Chem. 250,* 2968.