Himmelwright, R. S., Eickman, N. C., LuBien, C. D., & Solomon, E. I. (1980) *J. Am. Chem. Soc. 102*, 5378–5388.
Hintermann, G., Zatchej, M., & Hütter, R. (1985) *Mol. Gen. Genet. 200*, 422–432.
Huber, M., Hintermann, G., & Lerch, K. (1985) *Biochemistry 24*, 6038–6044.
Jolley, R. L., Evans, L. H., Makino, N., & Mason, H. S. (1974) *J. Biol. Chem. 249*, 335–345.
Joyce, C. M., & Grindley, N. D. F. (1984) *J. Bacteriol. 158*, 636–644.
Kieser, T., Hopwood, D. A., Wright, H. M., & Thompson, C. J. (1982) *Mol. Gen. Genet. 185*, 223–238.
Knowles, J. R. (1987) *Science (Washington, D.C.) 236*, 1252–1258.
Kunkel, T. A. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 488–492.
Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.
Lerch, K. (1976) *FEBS Lett. 69*, 157–160.
Lerch, K. (1981) *Met. Ions Biol. Syst. 13*, 143–186.
Lerch, K. (1983) *Mol. Cell. Biochem. 52*, 125–138.
Lerch, K., & Ettlinger, L. (1972) *Eur. J. Biochem. 31*, 427–437.
Lerch, K., Huber, M., Schneider, H.-J., Drexel, R., & Linzen, B. (1986) *J. Inorg. Biochem. 26*, 213–217.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.
Mandel, M., & Higa, A. (1970) *J. Mol. Biol. 53*, 159–162.
Mason, H. S. (1965) *Annu. Rev. Biochem. 34*, 594–634.
Miller, J. H. (1972) in *Experiments in Molecular Genetics*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Miyoshi, K., Huang, T., & Itakura, K. (1980a) *Nucleic Acids Res. 8*, 5491–5505.
Miyoshi, K., Miyaka, T., Hozumi, T., & Itakura, K. (1980b) *Nucleic Acids Res. 8*, 5473–5490.
Moore, S., & Stein, W. H. (1963) *Methods Enzymol. 6*, 819–831.
Peisach, J., & Blumberg, W. E. (1974) *Arch. Biochem. Biophys. 165*, 691–708.
Sakaguchi, U., & Addison, A. W. (1979) *J. Chem. Soc., Dalton Trans.*, 600–608.
Sanger, F., Nicklen, S., & Coulsen, A. R. (1979) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463–5468.
Schoot Uiterkamp, A. J. M., & Mason, H. S. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 993–996.
Shibahara, S., Tomita, Y., Sakakura, T., Nager, C., Chaudhuri, B., & Müller, R. (1986) *Nucleic Acids Res. 14*, 2413–2427.
Skotland, T., & Ljones, T. (1979) *Eur. J. Biochem. 94*, 145–151.
Solomon, E. I. (1981) in *Copper Proteins* (Spiro, T. G., Ed.) pp 42–108, Wiley-Interscience, New York.
Thompson, C. J., Ward, J. M., & Hopwood, D. A. (1980) *Nature (London) 286*, 525–527.
Thompson, C. J., Ward, J. M., & Hopwood, D. A. (1982) *J. Bacteriol. 151*, 668–677.
Yanisch-Perron, C., Vieira, J., & Messing, J. (1985) *Gene 33*, 103–119.
Zoller, M. J., & Smith, M. (1983) *Methods Enzymol. 100*, 468–500.

# Disulfide-Linked Dimer of Oncomodulin: Comparison to Calmodulin†

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ABSTRACT: Oncomodulin, an oncofetal $Ca^{2+}$-binding protein, contains a single Cys residue in position 18 of its primary structure. The reactivity of the Cys-18 thiol has been probed with 5,5′-dithiobis(2-nitrobenzoate) ($NbS_2$). The kinetics of the reaction indicate that the thiol group is ~10-fold more reactive in the presence of $Ca^{2+}$ than in its absence. Evidence presented here shows that oncomodulin can dimerize by intermolecular disulfide formation via the Cys-18 thiol. The kinetics of dimer formation indicate that the second-order rate constant for this reaction is ~6-fold higher than that observed for the reaction of the Cys-18 thiol with $NbS_2$, possibly indicating that intermolecular electrostatic interactions precede disulfide formation. The disulfide-linked dimer of oncomodulin appears to be more similar to calmodulin than oncomodulin since the dimer displayed "calmodulin-like" affinity for the amphiphilic peptide melittin. In addition, oncomodulin dimer was shown to activate two calmodulin-dependent enzymes, cyclic nucleotide phosphodiesterase and calcineurin phosphatase, with the activity constants of 63 and 1 nM, respectively, indicating that these enzymes have different domain contact requirements for activation.

Oncomodulin is a $Ca^{2+}$-binding protein ($M_r$ 11 700) originally isolated from rat Morris hepatomas (MacManus, 1979). It has since been detected in tumors from mice, guinea pigs, and humans (MacManus et al., 1983, 1984). The detection of this protein in the rat placenta indicates that oncomodulin is oncodevelopmental in origin; that is, it is expressed in early development and upon transformation (Brewer & MacManus, 1985; MacManus et al., 1985).

The primary structure of oncomodulin (ONC)[1] shows extensive homology to the β-parvalbumin subclass of the troponin

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[1] Abbreviations: $NbS_2$, 5,5′-dithiobis(2-nitrobenzoate); ONC, oncomodulin; ONC-d, disulfide-linked oncomodulin dimer; CaM, calmodulin; PDE, cyclic nucleotide phosphodiesterase; CaN, calcineurin; PV, parvalbumin; TnC, troponin C; DTT, dithiothreitol; PNPP, *p*-nitrophenyl phosphate; PAGE, polyacrylamide gel electrophoresis, HPSEC, high-performance size-exclusion chromatography.

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superfamily (MacManus et al., 1983; Heizmann, 1987). However, the similarities between oncomodulin and β-parvalbumins do not appear to extend to their tertiary structures since ONC was shown to undergo a $Ca^{2+}$-specific conformational change, in contrast to parvalbumin where identical perturbations were affected both by $Mg^{2+}$ and by $Ca^{2+}$ (MacManus et al., 1984a; Henzel et al., 1986; Williams et al., 1987). In addition, the $Ca^{2+}$-induced conformational change in oncomodulin appears not to be limited to the vicinity of the $Ca^{2+}$-binding sites as it was detected by a conformation probe covalently attached to the opposite face of the protein from the $Ca^{2+}$-binding sites (Mutus et al., 1985a).

The physiological role of oncomodulin is as yet unclear. However, several lines of evidence such as the activation of the calmodulin-dependent enzymes cyclic nucleotide phosphodiesterase (MacManus, 1981; Mutus et al., 1985b) and nuclear protamine kinase (MacManus & Whitfield, 1983) and the fact that oncomodulin can substitute for calmodulin in stimulating DNA synthesis in $Ca^{2+}$-deprived cells (Boynton et al., 1982) point to a calmodulin-like modulatory role for this tumor protein. In spite of this evidence, a modulatory role for oncomodulin is questionable in view of the fact that the concentrations of this protein required to give half-maximal stimulation are too high to be physiologically significant (Mutus et al., 1985b).

In this report, we provide evidence for the dimerization of oncomodulin via disulfide bridge formation through the oxidation of its Cys-18 thiol. Furthermore, the present study indicates that the oncomodulin dimer and calmodulin share common secondary structural features as inferred from a comparison of the affinities of these proteins for melittin, a cytotoxic peptide from bee venom which is generally thought to possess the structural features of the "calmodulin-binding domain" of target proteins (Comte et al., 1983; Maulet & Cox, 1983; Cox et al., 1985; Caday & Steiner, 1986). Apart from the apparent structural similarities, this study indicates that functionally the oncomodulin dimer is more calmodulin-like in that it can stimulate the activity of two enzymes with similar affinities to those observed for calmodulin.

The findings of the present study indicating functional and structural similarities between oncomodulin dimer and CaM are potentially very significant in implicating oncomodulin dimer and not oncomodulin as the biologically active molecule.

## Materials and Methods

*Materials.* Oncomodulin was isolated from rat Morris hepatoma 5123tc (MacManus, 1980). Carp β-parvalbumin was purified from skeletal muscle (Haiech et al., 1979). Bee venom melittin was purchased from Sigma and further purified by affinity chromatography on a column of calmodulin–Sepharose (Sharma et al., 1980). Calmodulin and cyclic nucleotide phosphodiesterase (PDE) were isolated from bovine brain and bovine heart, respectively, by established procedures (Sharma & Wang, 1979). Dansylcalmodulin was prepared according to Kincaid et al. (1982). Calcineurin was isolated from bovine brain by the method of Sharma et al. (1983). PDE and CaN preparations were purified further on a column of organomercurial Sepharose (Sluyterman & Wijdenes, 1970). Prior to this chromatography, the enzyme samples were dialyzed against citrate buffer (0.1 M; containing 20% v/v glycerol, pH 6.8) in order to remove the 2-mercaptoethanol. The enzymes bound onto the organomercurial Sepharose column were washed with buffer (Tris-HCl, 50 mM; KCl, 0.3 M; and EGTA, 0.1 mM, pH 7.4) containing 2-mercaptoethanol (10 mM). In the case of CaN, the elution buffer contained $MgCl_2$ (2.0 M). The eluted enzymes were dialyzed against citrate/glycerol prior to their titration by oncomodulin. Organomercurial Sepharose chromatography was also utilized in the separation of oncomodulin dimer from oncomodulin: the dimer which contains a disulfide bridge is not bound to this column whereas oncomodulin, with its free thiol, is attached via mercaptide formation. The latter can be eluted from the column with a buffer containing 2-mercaptoethanol. The purity of the enzymes and $Ca^{2+}$-binding proteins was established by SDS–PAGE (Laemmli, 1970); that of melittin was determined by HPSEC (Bio-Sil TSK 125) in buffer (Tris-HCl, 50 mM; KCl, 150 mM, pH 6.8) containing 4 M urea. Melittin-conjugated Sepharose (Melex) was prepared according to Cox et al. (1985). Other reagents and biochemicals used in this study were purchased from Sigma.

*Protein Determinations.* Protein concentrations were determined by the method of Bradford (1976). A correction factor for each protein was formulated by comparing the values obtained by this method to those from the reported extinction coefficients.

*Enzyme Assays.* Cyclic nucleotide phosphodiesterase activity was determined as described by Sharma and Wang (1979). The calcineurin phosphatase was assayed directly with *p*-nitrophenyl phosphate (PNPP) as the substrate (Pallen & Wang, 1983). The assay mixture (500 μL) contained CaN (40 μg), Mn(II) and $Ca^{2+}$ (1 mM), and PNPP (10 mM) in Tris-HCl (50 mM) and KCl (150 mM), pH 7.5 (buffer A). The reaction was stopped by the addition of $K_2HPO_4$ (10% w/v), subsequent to incubation at 30 °C for 10 min. The amount of product, *p*-nitrophenol, was determined spectrophotometrically (extinction coefficient = 18 000 $M^{-1}$ $cm^{-1}$ at 405 nm) (Kincaid & Vaughan, 1986). Free $Ca^{2+}$ in the $Ca^{2+}$ titration was obtained by using EGTA buffering (Mutus et al., 1985a).

*Thiol Titrations.* Freshly reduced samples of ONC (1 mg) were stripped from excess reducing agent by chromatography on a column of Sephadex G-25 which was preequilibriated with buffer A. The fractions containing protein were pooled and rapidly transferred to a cuvette. The absorbance of the samples were then followed at 412 nm subsequent to the addition of $NbS_2$ (50-fold molar excess).

In the dimerization study, the SH content was determined by an identical procedure except that $NbS_2$ stock solutions contained SDS to give a final in cuvette concentration of 0.1%. This was done in order to ensure that the decreases in thiol content with respect to time was due to oxidation and not to their inaccessibility to the reagent.

*Melex Capacity.* The capacity of Melex for DNS-CaM was determined by the application of an excess of DNS-CaM (500 μg) to a column (0.75 × 1 cm) of Melex (500-μL packed volume) equilibriated in buffer A containing 4 M urea (buffer B). This column was stoppered at both ends and placed horizontally on a shaker. The column was allowed to pack after shaking at low speed, at room temperature, for 15 min. The unbound DNS-CaM was eluted by washing with 20 mL of the equilibrating buffer. At this point, the bound DNS-CaM could be visualized with a hand-held UV lamp. The DNS-CaM was then eluted with buffer C (Tris-HCl, 50 mM; KCl, 600 mM; and EGTA, 1 mM, pH 7.4). Under these conditions, Melex had a capacity of 0.02 μmol of DNS-CaM/mL of packed gel.

*DNS-CaM Displacement Assay.* This is a modification of the [$^3$H]CaM displacement assay first introduced by Cox et al. (1985). DNS-CaM (0.1 μM) was added to Melex suspensions, in buffer B (which had a capacity of binding 100% of the added DNS-CaM), containing varying amounts of the

competing proteins (CaM, ONC, ONC-d, TnC, and PV). The total volume of the mixture contained in 1.5-mL airfuge tubes was 1.2 mL. The assay mixture, after being shaken for 30 min at 25 °C, was centrifuged in an Eppendorf airfuge. The supernatant (800 μL) was withdrawn, and the amount of free DNS-CaM was determined fluorometrically on a Shimadzu RF-540 spectrofluorometer (excitation 350 nm, emission 505 nm).

The fraction of Melex-bound DNS-CaM ($f_b$) was calculated from eq 2 where $F_B$ corresponds to a sample in which 100%

$$F_T = F_F + F_B \quad (1)$$

$$f_b = (F - F_B)/F_T \quad (2)$$

of the DNS-CaM is bound to Melex (i.e., a sample devoid of competing protein). $F_F$ represents a sample where none of the DNS-CaM is bound to Melex. This value was obtained by incubating DNS-CaM in Melex equilibrated with buffer C (EDTA). The supernatant of this sample, subsequent to centrifugation, was made 5 mM in $Ca^{2+}$ by the addition of 10 μL of a 0.5 M solution of $Ca^{2+}$ in buffer B, in order to avoid differences in emission intensity between $Ca^{2+}$-free and $Ca^{2+}$-saturated forms of DNS-CaM. The total fluorescence, $F_T$, therefore equals $F_F + F_B$. $F$ represents the fluorescence of a sample containing constant amounts of Melex and DNS-CaM in the presence of a given amount of competing protein. Nonspecific interactions between the insoluble support (CNBr-activated Sepharose) and DNS-CaM can be ruled out as the values of $F_T$ were identical whether it was calculated as above (i.e., DNS-CaM plus Melex in buffer C) or from a sample in which DNS-CaM was added to buffer B alone.

The competing proteins did not interfere with the DNS-CaM fluorescence intensity since the slopes of plots of DNS fluorescence intensity vs DNS-CaM concentration (corresponding to a concentration range between $F_F$ and $F_B$) were not affected by increasing amounts of the competing proteins.

S-Carboxymethylation with iodoacetic acid of the Cys-18 thiol of ONC was performed according to the procedure of Hirs (1967). PAGE was performed in the presence of either lauryl sulfate (10%) or urea (4 M) on 10% or 15% slab gels and run on a Bethesda Research Laboratories V12 vertical slab apparatus according to the method of Laemmli (1970).

## Results

*Oncomodulin Thiol Reactivity.* ONC contains a single thiol group in position 18 of its primary structure. The reactivity of this thiol was probed with the thiol-specific reagent $NbS_2$, in the presence and absence of $Ca^{2+}$. The thiol group appears to be more reactive toward $NbS_2$ in the $Ca^{2+}$-induced conformation: the second-order rate constant, $k_2$, estimated from the pseudo-first-order plots of the time course (Figure 1) in the presence of $Ca^{2+}$, 240 ± 5 $M^{-1}$ $min^{-1}$, was 10-fold larger than that estimated for the reaction in the absence of this cation (EGTA), 24 ± 5 $M^{-1}$ $min^{-1}$.

ONC is thought to dimerize via intermolecular disulfide formation through the oxidation of its Cys-18 thiol. The evidence for this is presented in Figure 2; samples of this protein, extensively dialyzed against DTT, anomalously run on SDS–PAGE with molecular weight of ~16 000 (Figure 2a). However, ONC samples devoid of thiol reducing agents, when left at room temperature, were converted to a larger $M_r$ ~27 000 species in a time-dependent manner (Figure 2b,c). The larger molecular weight band could then be converted to the lower molecular weight band by the exposure of the former to DTT (20 mM) in the presence of SDS (0.1%) (Figure 2d).

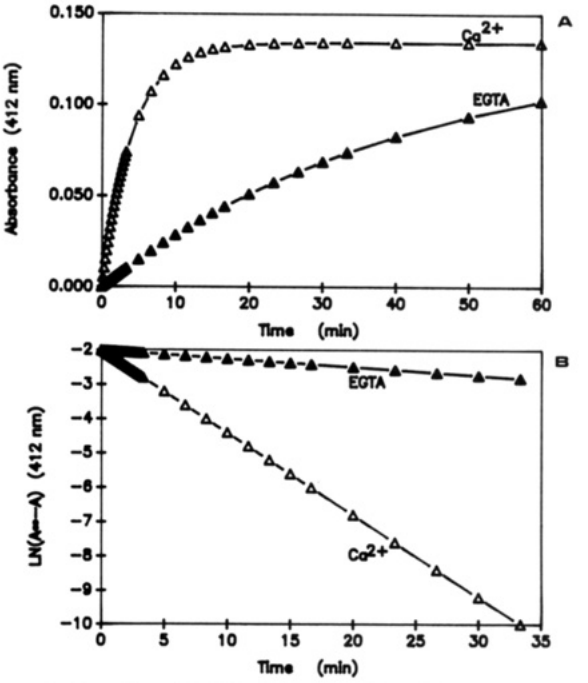


FIGURE 1: Reaction of ONC with $NbS_2$. (Panel A) Time course of the reaction of ONC (10 μM), $NbS_2$ (0.5 mM), and $Ca^{2+}$ (1 mM) (△) or EGTA (1 mM) (▲) in buffer A, 30 °C. (Panel B) Pseudo-first-order plot of the data in panel A. The solid line represents the theoretical best fit of the data to the first-order rate equation by the Simplex method (Noggle, 1985).

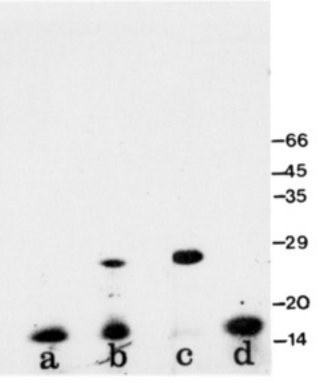


FIGURE 2: SDS–PAGE analysis of ONC forms in buffer A containing DTT (1 mM). Lane a, ONC samples incubated at room temperature in the absence of reducing agents for 2 h; lane b, 22 h; lane c, the 22-h sample subsequent to reduction in 0.1% SDS, 80 °C, for 15 min; lane d, molecular weight standards; bovine serum albumin (66 200), glyceraldehyde-3-phosphate dehydrogenase (35 000), ovalbumin (42 700), carbonic anhydrase (28 900), soybean trypsin inhibitor (20 100), and lysozyme (14 300). Each lane contained ~15 μg of protein.

The dimerization of ONC via disulfide bond formation is described by eq 3. Therefore, the second-order rate constant

$$\text{ONCSH} \xrightarrow{(0)} \text{ONCS–SONC} \quad (3)$$

for the dimerization can be indirectly determined by measuring the decrease in the concentration of free thiol with respect to time. The results of such an experiment are presented in Figure 3, where aliquots of ONC were removed from an incubation mixture (containing either $Ca^{2+}$, 1 mM, or EGTA, 1 mM) and titrated for free thiol content with $NbS_2$. ONC appears not to dimerize in the absence of $Ca^{2+}$ as there was no significant decrease in the thiol content for up to 4 h when incubated with EGTA. Even at the end of 22 h, only ~5% of the sample was oxidized. In the presence of $Ca^{2+}$, the

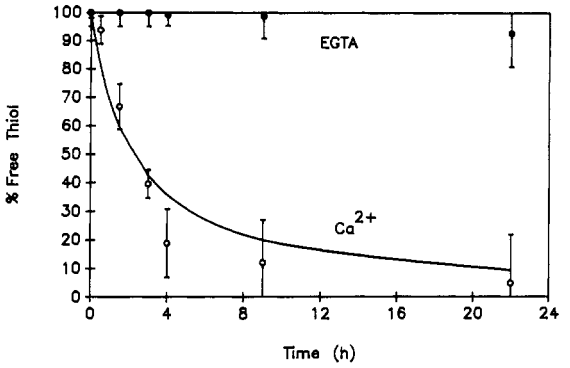


FIGURE 3: Time course of the oxidation of ONC thiol. ONC (35.6 μM), subsequent to the removal of DTT by Sephadex G-25 chromatography, was incubated at 30 °C. At various time intervals, samples (100 μL) were withdrawn and added to a cuvette which contained $NbS_2$ (0.5 mmol/L), SDS (0.1%), and buffer A to a total volume of 1.0 mL. The absorbance increase at 412 nm was followed until the absorbance change was zero. The solid line represents the theoretical best fit of the data to the second-order rate equation $1/A - 1/A_0 = kt$) by the Simplex method (Noggle, 1985).

oxidation was comparatively rapid. The $k_2$ estimated from a fit of the data to the second-order rate equation was 1500 ± 230 $M^{-1}$ $min^{-1}$. This rate contant is ~6-fold higher than the $k_2$ estimated for the reaction of $NbS_2$ with the free thiol of ONC (Figure 2), possibly indicating that ONC self-associates prior to disulfide bond formation.

*Oncomodulin–Melittin Interactions.* Superficially, the disulfide-linked dimer of ONC (ONC-d) would more closely resemble CaM in that the dimer would have four $Ca^{2+}$-binding sites and the two metal ion binding domains would be linked via long peptide segments. In order to test this hypothesis, ONC and ONC-d were compared to CaM with respect to their secondary structural and regulatory properties. The secondary structural similarities between these proteins were probed by comparing their binding affinities to melittin, which has been shown to possess the minimum secondary structural features of the CaM-binding domain of target enzymes (Comte et al., 1983; Maulet & Cox, 1983; Cox et al., 1985; Caday & Steiner, 1986). Therefore, proteins displaying affinities for melittin similar to that observed for CaM would likely possess the secondary structural features of the target protein binding domain of CaM.

Evidence for $Ca^{2+}$-dependent, high-affinity interactions between ONC-d and melittin is presented in Figures 4 and 5. These experiments were performed in the presence of 4 M urea in order to avoid low affinity, nonspecific interactions previously observed between melittin and CaM (Maulet & Cox, 1983). The elution profiles of samples of ONC and ONC-d applied onto a column of melittin, covalently attached to Sepharose (Melex) are presented in Figure 4. Nearly all (>90%) of the ONC-d sample was bound to the melittin column in the presence of $Ca^{2+}$. The adsorbed protein is then eluted upon chelation of the $Ca^{2+}$ by EGTA. In the case of ONC, ~75% of the sample was not retained by the column. The ~25% of the protein which is bound to Melex is thought to represent the fraction of ONC which oxidized during the incubation period (30 min). The dimerization is likely to occur more rapidly as these experiments were performed under partially denaturing conditions (4 M urea). Additional evidence for melittin–ONC-d interactions was obtained from changes in the intrinsic melittin Trp fluorescence. The emission spectrum of melittin which was unaltered by ONC-d in the presence of EGTA was enhanced and blue-shifted in the presence of ONC-d and $Ca^{2+}$ (data not shown). These spectral changes were comparable to those previously reported

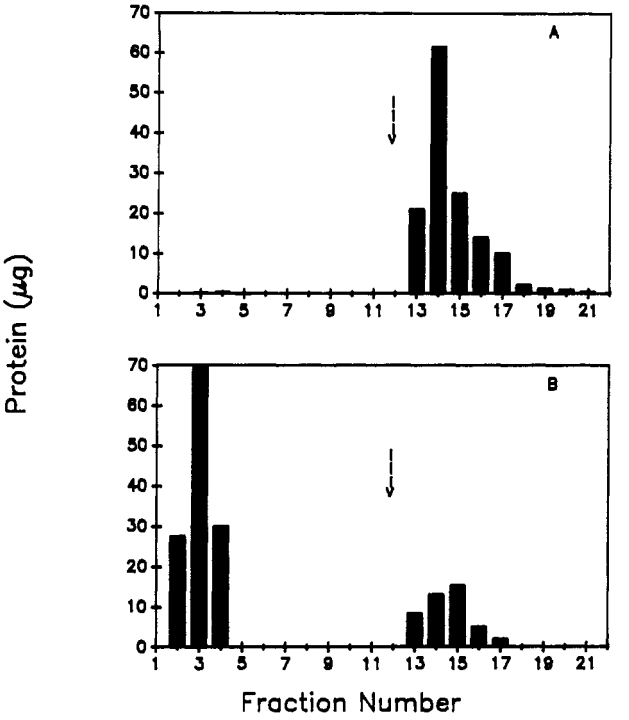


FIGURE 4: Melittin–Sepharose elution profiles of ONC and ONC-d. (Panel A) ONC-d (170 μg) or (panel B) ONC (180 μg) was added to 0.5 mL of a melittin-Sepharose gel suspension in buffer A containing $Ca^{2+}$ (0.1 mM) and urea (4 M) and placed in a horizontal shaker for 30 min. The gel was then packed into a column (0.5 × 5 cm) and eluted with the same buffer. At the point indicated by the arrow, the elution buffer was changed to buffer A containing EGTA (0.1 mM) and urea (4 M).

Scheme I

$$\begin{array}{ccc} M + \text{DNS-CaM} & \overset{K_c}{\rightleftharpoons} & M\bullet\text{DNS-CaM} \\ + & & \\ \text{Comp} & & \\ \Updownarrow K_{comp} & & \\ M\bullet\text{Comp} & & \end{array}$$

for melittin–CaM (Maulet & Cox, 1983) and melittin–S100b (Baudier et al., 1987). The dissociation constants between ONC, ONC-d, and melittin were estimated by the labeled CaM displacement assay procedure using Melex (Cox et al., 1985). In these experiments, the fluorescent-labeled dansyl derivative of CaM (DNS-CaM) (0.1 μmol/L) was incubated with Melex, with enough capacity to bind all of the DNS-CaM. The equilibria describing the competitive binding are shown in Scheme I where DNS-CaM and the competing protein, Comp, bind to Melex, M, with the dissociation constants $K_c$ and $K_{comp}$, respectively. An expression for the fraction of DNS-CaM bound to Melex, $f_B$, at a given competing protein concentration, derived from Scheme I, is

$$f_B = [\text{DNS-CaM}]/[\text{DNS-CaM}] + K_c(1 + [\text{Comp}]/K_{comp}) \quad (4)$$

According to eq 4, in the absence of competing ligand at DNS-CaM concentrations ~100-fold higher than $K_c$, nearly all of the DNS-CaM will be bound. On the other hand, in the presence of competing proteins, displaying affinity for Melex similar to that observed for DNS-CaM (i.e., $K_c = K_{comp}$), 50% of the bound DNS-CaM will be displaced when the concentration of the competing protein equals that of the labeled CaM. This is precisely what was observed experimentally when native CaM was used as the competing protein (Figure 5): ~50% of the DNS-CaM was displaced when the

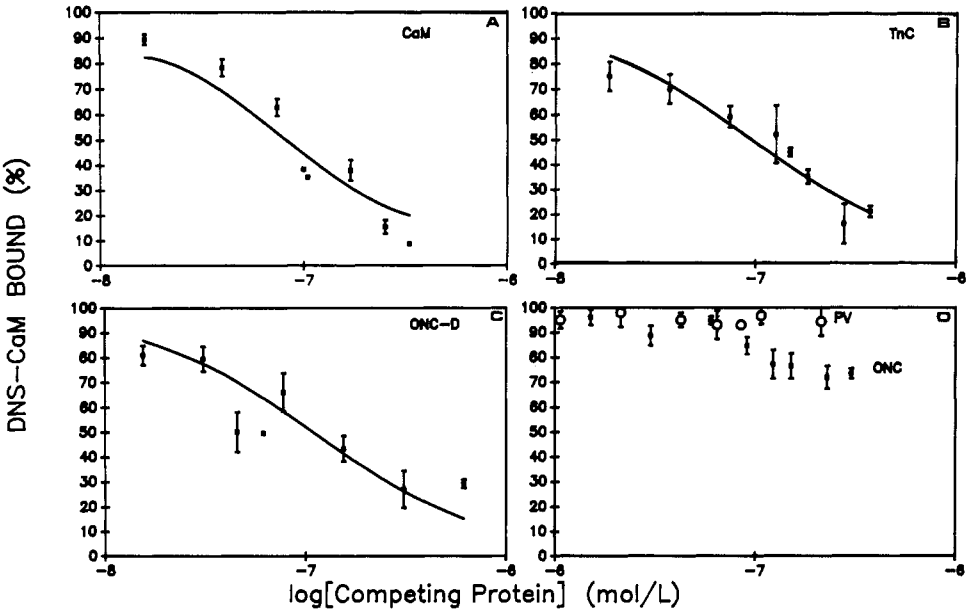


FIGURE 5: DNS-CaM displacement assay. DNS-CaM (0.1 μmol/mL) plus melittin–Sepharose suspension (0.4 mL), with enough capacity to bind 100% of the DNS-CaM, plus increasing amounts of the competing proteins [CaM, panel A; TnC, panel B; ONC-d, panel C; ONC and carp PV, panel D (total volume = 1.0 mL)] was incubated for 30 min in a shaker in airfuge tubes (1.5 mL). Subsequent to the incubation, the tubes were centrifuged, and supernatant (0.8 mL) was withdrawn and quantitated fluorometrically ($\lambda_{ex}$ = 350 nm, $\lambda_{em}$ = 505 nm) for the amount of free DNS-CaM. Fraction bound was then calculated according to eq 2. The solid lines represent the theoretical best fit of the data to eq 4 by the simplex method (Noggle, 1985). The $K_{comp}$ values estimated in this manner for CaM, TnC, and ONC-d were 0.8, 0.9, and 1.1 nM, respectively. The ONC and PV data could not be fitted as these proteins did not displace significant amounts of DNS-CaM. The data points represent the average of three determinations. The error bars represent the standard error.

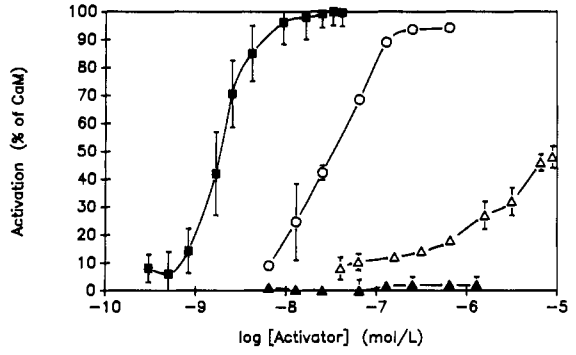


FIGURE 6: Titration of bovine heart PDE activity with CaM (■), ONC-d (○), ONC (△), and *S*-(carboxymethyl)-ONC (▲). The PDE concentration was 0.1 nmol/L. Activation is defined as the activity at a given concentration of activator divided by the amount of activity produced by excess amounts of CaM, expressed as a percentage. PDE was activated 5.5- and 5-fold by CaM and ONC-d, respectively. The data points represent the average of four determinations. The error bars represent the standard error.

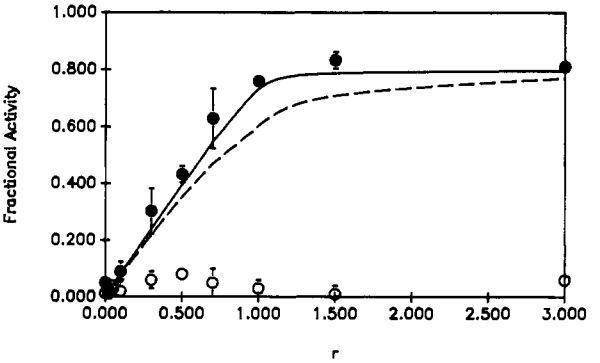


FIGURE 7: Titration of CaN phosphatase activity with ONC-d and ONC. Fractional activity is expressed as the activity at a given concentration of ONC-d (●) or ONC (○) divided by the amount of activity produced by excess amounts of CaM. CaN was activated 4.3- and 3.5-fold by CaM and ONC-d, respectively. The CaN concentration was 100 nM. The solid line represents the theoretical best fit of the data to the Richards and Vithayathil (1959) equation by the Simplex method (Noggle, 1985). The $K_d$ between CaN and ONC-d estimated in this manner was 1 nM. The dashed line represents the theoretical line calculated for a $K_D$ of 10 nM. The data points represent the average of four determinations. The error bars represent the standard error.

[CaM] = [DNS-CaM] = $1 \times 10^{-7}$ M. Fit of the displacement data, with native CaM, to eq 4 by the simplex method (Noggle, 1985) yielded a $K_{comp}$ of ~0.6 nM, in agreement with previous estimates (Comte et al., 1983). The displacement profile with troponin C, which can substitute for CaM in activating some target enzymes (Marcum et al., 1978; Picton et al., 1980), was nearly superimposable on that of CaM ($K_{comp}$ = 0.9 nM). On the other extreme, carp parvalbumin was unable to displace DNS-CaM to any appreciable extent even at concentrations 10-fold higher than that of DNS-CaM, indicating that this protein does not form high-affinity interactions with melittin, as was previously shown (Comte et al., 1983). The displacement profiles of ONC and ONC-d were dramatically different; at concentrations of ONC and ONC-d which equalled that of the labeled CaM ($1 \times 10^{-7}$ M), the fractional saturation of Melex was 0.85 and 0.5, respectively. A $K_{comp}$ of 1.1 nM was estimated from the fit of the ONC-d displacement data to eq 4, whereas the $K_{comp}$, estimated from the extrapolation of the ONC displacement data, was $>1 \times 10^{-6}$ M. These results suggest that a high-affinity melittin-binding domain is formed upon dimerization on ONC via intermolecular disulfide formation. If this melittin-binding domain is similar to the CaM target protein binding domain, as suggested by the magnitude of the ONC-d–melittin dissociation constant, then the ONC dimer should display higher apparent affinity for CaM-dependent target enzymes in comparison to ONC. In order to test this hypothesis, two CaM-dependent enzymes, bovine heart cyclic nucleotide phosphodiesterase (PDE) and brain calcineurin phosphatase (CaN), were tested with respect to ONC and ONC-d dose-dependent effects on their catalytic activity.

*Functional Comparison of ONC and ONC-d to CaM.* The

activity of PDE and CaN as a function of ONC, ONC-d, and CaM concentration is presented in Figures 6 and 7, respectively. ONC-d stimulated bovine heart PDE to ~90% of that observed with CaM, with an estimated $K_{activation}$ of 63 nM. ONC also activated the enzyme but with a much higher $K_{activation}$ (~10 μM). This low-affinity activation observed with ONC is thought to result from a fraction of the protein that has been dimerized as ONC which was S-carboxymethylated with iodoacetate failed to activate PDE (Figure 6). In the case of CaN, only ONC-d was able to stimulate enzyme activity (Figure 7). A $K_d$ of ~1 nM was estimated from plots of fractional activity vs mole ratio (ONC-d/CaN) (Richards & Vithayathil, 1959). The activation of both enzymes was $Ca^{2+}$ dependent. The affinity of ONC-d for $Ca^{2+}$ was estimated to be 1.2 and 1.5 μM from the $Ca^{2+}$ dose-dependent activation of PDE and CaN, respectively (data not shown). It is apparent from these results that the melittin-binding domain of ONC-d has similar features to the CaM target protein binding domain in that this protein was able to activate one of the enzymes tested, CaN, with an estimated $K_d$ that is only 10 times higher than the value reported for CaM (Hubbard & Klee, 1986).

## Discussion

Oncomodulin has the distinction of being the only $Ca^{2+}$-binding protein that is clearly associated with a disease state as this protein is not found in any normal adult tissue. It is expressed early in development and upon cellular transformation. Therefore, the elucidation of its physiological role is of primary importance especially since ONC displays some of the properties characteristic of the regulatory members of the troponin C superfamily such as the ability to undergo large, $Ca^{2+}$-specific, conformational changes (Mutus et al., 1985a; MacManus et al., 1984a; Henzel et al., 1986; Williams et al., 1987). In addition, ONC has been shown to substitute for CaM in stimulating the activity of a few CaM-dependent enzymes (MacManus, 1981; Mutus et al., 1985b). However, ONC cannot be implicated as a regulatory protein based on the evidence to date, since the number of ONC-dependent enzymes/processes is very small and the amounts of this protein required for the stimulatory response are too high to be physiologically relevant (Mutus et al., 1985b).

In this paper, we have presented evidence for a related molecular form of ONC, the disulfide-linked ONC dimer. Since the ONC dimer is formed via the oxidation of the Cys-18 thiol of ONC, we first examined the reactivity of this functional group with $NbS_2$. These studies revealed that the ONC thiol is ~10-fold more reactive toward $NbS_2$ in the presence of $Ca^{2+}$ than in its absence (EGTA). This is exactly the opposite of what has been reported for the reaction of carp PV with this reagent (Donato & Martin, 1974), once again indicating that although ONC and PV share many homologous regions, these proteins are dynamically distinct. The greater reactivity of the Cys-18 thiol in the presence of $Ca^{2+}$ suggests that this functional group is more exposed to solvent in the $Ca^{2+}$ conformation of ONC, as was indicated in a recent study with the thiol-specific reagent *N*-dansylaziridine (Mutus et al., 1985a).

The studies were then extended to include the kinetics of disulfide bridge formation between ONC molecules. The second-order rate constant for this reaction was ~6-fold higher than that observed for the reaction of the Cys-18 thiol of ONC with $NbS_2$. This result suggests that the ONC Cys-18 thiol is in a more favorable orientation for intermolecular disulfide formation, a possible indication that electrostatic interactions between ONC molecules, which bring Cys-18 thiols in close proximity, precede disulfide bridge formation.

The recently elucidated three-dimensional structures of CaM and TnC have revealed that these proteins are dumbbell-shaped; the two nearly globular regions, each of which contains two $Ca^{2+}$-binding domains, are connected by a long helical peptide segment (Herzberg & James, 1985; Babu et al., 1985). In comparison, ONC can be thought of as a half of a CaM molecule since this protein contains only two $Ca^{2+}$-binding domains. Furthermore, a globular shape for this molecule is probable in view of the extensive homology between ONC and PV (MacManus et al., 1983; Heizmann, 1987). Therefore, upon dimerization, ONC would more closely resemble CaM in that the two globular heads containing the $Ca^{2+}$-binding sites would now be linked by a long irregular arrangement of helical segments. The present study indicates that ONC-d does appear to share some secondary structural features with CaM. This conclusion is based on the fact that ONC-d was shown to interact with high affinity with melittin, a small protein which has its 26 consistuent amino acids arranged in two α-helical segments, thus giving an overall shape of a bent rod. In addition, this bent rod contains polar residues on one face and basic residues on the other (Terwilliger & Eisenberg, 1982). Therefore, the melittin interaction domain is likely an acidic, amphiphilic, α-helical region. The fact that CaM-like affinities (in the nanomolar range) were estimated from the displacement assay for ONC-d suggests the presence of a similar domain on this tumor protein. In addition, the >100-fold lower affinity estimated from displacement assays for ONC would indicate that the melittin-binding domain is formed upon dimerization.

Basic amphiphilic peptides such as melittin have been used as models of the CaM-binding domain of target proteins principally because these peptides display affinities for CaM (1–3 nM) that are nearly identical with $K_d$ or $K_{activity}$ values estimated between CaM and target enzymes such as PDE (1 nM) (Kuznicki et al., 1984), phosphorylase kinase (10 nM) (Picton et al., 1980), CaN (<0.1 nM) (Hubbard & Klee, 1986), and myosin light chain kinase (1.3 nM) (Malencik & Anderson, 1982). Therefore, interactions of similar affinity between ONC-d and melittin would suggest the presence of a CaM-like target protein binding domain on ONC-d. If this is the case, then ONC-d should activate CaM-dependent enzymes at lower, more physiologically relevant concentrations than observed with ONC. This was precisely what was observed when, in this study, two CaM-dependent enzymes (CaN and PDE) were examined with respect to ONC- and ONC-d-dependent effects on their catalytic activity; ONC had no apparent effect on CaN activity whereas ONC-d activated this enzyme to ~80% of the CaM-stimulated activity with an estimated $K_d$ of 1 nM. With PDE, both ONC and ONC-d enhanced the activity; however, the $K_{activation}$ estimated for the dimer was 63 nM whereas that estimated for ONC was ~10 μM.

The low-affinity activation of PDE with ONC is thought to be caused by a small fraction of the protein that is dimerized as ONC samples which had their free thiols blocked by S-carboxymethylation failed to activate the enzyme. The results presented here indicate that ONC-d interacts with CaN with 63-fold higher affinity in comparison to PDE. An explanation for these observations is thought to be related to the recent findings of several workers (Newton et al., 1984; Klevit & Vanaman, 1984; Putkey et al., 1986; Ni & Klee, 1985; Hansen & Beavo, 1986) which suggest multiple modes of interaction of CaM with its target proteins. On the basis of these studies, the target enzymes have been classified into two types. The type I enzymes which include PDE and myosin light-chain

kinase are thought to bind to CaM through multiple interactions involving two or more domains (Ni & Klee, 1985), whereas type II enzymes which include CaN and multiprotein kinase are thought to interact at only one domain near the COOH terminal of CaM (Ni & Klee, 1985). The present study indicated that ONC-d interacts with melittin with $K_d$s in the nanomolar range and thus should display similar affinities for CaM-dependent enzymes. The fact that this turned out to be the case with CaN, and not with PDE, would suggest that melittin in a good model for type II enzymes like CaN where activation is brought about as a result of a single interaction between the activator and the target enzyme. However, melittin does not appear to be a good model for type I enzymes like PDE where the activation is brought about as a consequence of multiple interactions between the target and the regulator. In fact recently Cachia et al. (1986) have used similar arguments to point out that amphiphilic peptides do not make good models for CaM–target protein interactions involving multiple domain contacts.

In this paper, we have presented evidence for the in vitro formation of a disulfide-linked oncomodulin dimer which more closely resembles CaM with respect to its melittin-binding affinity and its apparent $K_{activation}$ toward two CaM-dependent enzymes. Therefore, it is very tempting to speculate that ONC-d and not ONC is the biologically active molecule. Thus, a search for ONC-d-dependent processes would be the key to elucidating the physiological role of this tumor protein. In order for ONC-d to exist in sufficiently high concentrations in the cell, two criteria must be met: the dimerization reaction must be rapid, and once the dimer forms it must be stable to reduction. The results presented here do indicate that the dimer forms rapidly with a $k_2$ of $\sim$1500 $M^{-1}$ $min^{-1}$. However, ONC-d is not expected to exist intracellularly as the cytosol is a highly reducing environment. This is brought about by nanomolar levels of glutathione. In addition, the fact that ONC is expressed in tumors makes an intracellular role for ONC-d even less plausible as tumor tissues have recently been shown to contain glutathione levels that are 2–8-fold higher than normal (Lee et al., 1987; Russo et al., 1986; Murray et al., 1987). In view of these facts, the search for ONC-d-dependent processes should focus on the extracellular environment.

**Registry No.** PDE, 9040-59-9; CaN, 9025-75-6; Cys, 52-90-4; Ca, 7440-70-2.

## REFERENCES


Babu, Y. S., Sack, J. S., Greenbough, T. J., Bugg, L. E., Means, A. R., & Cook, W. J. (1985) *Nature (London) 315*, 37–40.

Baudier, J., Mochly-Rosen, D., Newton, A., Lee, S. H., Koshland, D. E., Jr., & Cole, R. D. (1987) *Biochemistry 26*, 2886–2893.

Boynton, A. L., MacManus, J. P., & Whitfield, J. F. (1982) *Exp. Cell Res. 138*, 454–458.

Bradford, M. (1976) *Anal. Biochem. 72*, 248–256.

Brewer, L. M., & MacManus, J. P. (1985) *Dev. Biol. 112*, 4950.

Cachia, P. J., Van Eyk, J., Ingraham, R. H., McCubbin, W. D., Kay, C. M., & Hodges, R. S. (1986) *Biochemistry 25*, 3553–3562.

Caday, C. G., & Steiner, R. F. (1986) *Biochem. Biophys. Res. Commun. 135*, 419–425.

Comte, M., Maulet, Y., & Cox, J. A. (1983) *Biochemistry 22*, 5680–5686.

Cox, J. A., Comte, M., Fitton, J. E., & De Grado, W. F. (1985) *J. Biol. Chem. 260*, 2427–2534.

Donato, H., & Martin, R. B. (1974) *Biochemistry 13*, 4575–4579.

Ellman, G. L. (1958) *Arch. Biochem. Biophys. 74*, 443–448.

Haiech, J., Derancourt, J., Pechere, J. F., & Demaille, T. G. (1979) *Biochimie 61*, 583–587.

Hansen, R. S., & Beavo, J. A. (1986) *J. Biol. Chem. 261*, 14636–14645.

Heizmann, C. W. (1987) *Methods Enzymol. 139*, 207–214.

Henzel, M. T., Hapak, R. C., & Birnbaum, E. R. (1986) *Biochim. Biophys. Acta 872*, 16–23.

Herzberg, O., & James, M. N. G. (1985) *Nature (London) 294*, 327.

Hirs, C. H. W. (1967) *Methods Enzymol. 11*, 199–203.

Kincaid, R. L., & Vaughan, M. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 1193–1197.

Kincaid, R. L., Vaughan, M., Osborne, J. C., Jr., & Tkachuk, V. A. (1977) *Biochemistry 16*, 1017–1024.

Klec, C. B., Krinks, M. H., & Hubbard, M. J. (1987) in *Calcium Binding Proteins in Health and Disease*, pp 481–490, Academic, New York.

Klevit, R. E., & Vanaman, T. C. (1984) *J. Biol. Chem. 259*, 15414–15424.

Kuznicki, J., Grabarek, Z., Brzeska, H., Drabikowski, W., & Cohen, P. (1984) *FEBS Lett. 130*, 141.

Laemmli, V. K. (1970) *Nature (London) 227*, 680–685.

Lee, F. Y. F.,, Allaluis-Turner, M. J., & Siemann, D. W. (1987) *Br. J. Cancer 56*, 33–38.

MacManus, J. P. (1979) *Cancer Res. 39*, 3000–3005.

MacManus, J. P. (1980) *Biochim. Biophys. Acta 625*, 296–304.

MacManus, J. P. (1981) *FEBS Lett. 126*, 245–249.

MacManus, J. P., & Whitfield, J. F. (1983) *Calcium Cell Funct. 4*, 411–440.

MacManus, J. P., Watson, D. C., & Yaguchi, M. (1983) *Biosci. Rep. 3*, 1071–1075.

MacManus, J. P., Szabo, A. G., & Williams, R. E. (1984) *Biochem. J. 220*, 261–268.

MacManus, J. P., Brewer, L. M., & Whitfield, J. F. (1985) *Cancer Lett. 21*, 309–315.

MacManus, J. P., Whitfield, J. F., & Stewart, D. J. (1986) *Cancer Lett. 21*, 309–315.

Malencik, D. A., & Anderson, S. R. (1982) *Biochemistry 21*, 3480–3486.

Marcum, J. M., Dedman, J. R., Brinkley, B. R., & Means, A. R. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 3771–3775.

Maulet, Y., & Cox, J. A. (1983) *Biochem. J. 209*, 264–273.

Murray, G. I., Burke, M. D. & Ewen, S. W. B. (1987) *Br. J. Cancer 55*, 605–609.

Mutus, B., Flohr, E. J., & MacManus, J. P. (1985a) *Can. J. Biochem. Cell Biol. 63*, 998–1002.

Mutus, B., Karuppiah, N., & MacManus, J. P. (1985b) *Biochem. Biophys. Res. Commun. 131*, 500–506.

Newton, D. L., Oldewurtel, M. D., Krinks, M. H., Shiloach, J., & Klee, C. B. (1984) *J. Biol. Chem. 259*, 4419–4426.

Ni, W. A., & Klee, C. B. (1985) *J. Biol. Chem. 260*, 6974–6981.

Noggle, J. (1985) in *Physical Holics Chemistry on a Microcomputer*, Little Brown and Co., Boston.

Pallen, C. J., & Wang, J. H. (1983) *J. Biol. Chem. 258*, 8550–8553.

Picton, C., Klee, C. B., & Cohen, P. (1980) *Eur. J. Biochem. 111*, 553.

Putkey, J. A., Draetta, G. F., Slaughter, G. R., Klee, C. B., Cohen, P., Stull, J. T., & Means, A. R. (1986) *J. Biol. Chem. 261*, 9896–9903.

Richards, F. M., & Vithayathil, P. J. (1959) *J. Biol. Chem. 234*, 1459-1465.
Russo, A., Degraff, W., Friedman, N., & Mitchell, J. B. (1986) *Cancer Res. 46*, 2845-2848.
Sharma, R. K., & Wang, J. H. (1979) *Adv. Cyclic Nucleotide Res. 10*, 187-198.
Sharma, R. K., Taylor, W. A., & Wang, J. H. (1983) *Methods Enzymol. 102*, 210-219.
Sluyterman, L. A. E., & Wijdenes, J. (1970) *Biochim. Biophys. Acta 200*, 593-595.
Terwilliger, T. C., & Eisenberg, D. (1982) *J. Biol. Chem. 257*, 6016-6022.
Williams, T. C., Corson, D. C., Sykes, B., & MacManus, J. P. (1987) *J. Biol. Chem. 262*, 6248-6256.

# Nus A Protein Affects Transcriptional Pausing and Termination in Vitro by Binding to Different Sites on the Transcription Complex

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ABSTRACT: We examined the in vitro concentration dependence of the effects of Nus A on transcription termination and pausing to determine if Nus A affects both pausing and termination in vitro by binding to a single site on the transcription complex. Nus A was shown to cause maximal increases of pausing at a concentration approximately equimolar to RNA polymerase. However, the effects of Nus A on termination require much higher Nus A concentrations than are required for pausing. It is therefore likely that the effects of Nus A on pausing and termination result from the binding of Nus A to different sites on the transcription complex. Since proteins that probably bind RNA nonspecifically were also shown to strongly reduce termination at a Rho-dependent terminator, Nus A may decrease Rho-dependent termination by binding nonspecifically to RNA. This proposal is consistent with most of the available data on the in vitro effects of Nus A and provides a mechanistic basis for previously unexplained details of Nus A caused decreases in Rho-dependent termination. We further speculate that most or all of the in vivo roles of Nus A may involve the enhancement of pausing.

Nus A protein of *Eshcerichia coli* is a transcription factor that causes decreased termination at Rho-dependent terminators, increased termination at a Rho-independent terminator, and increased pausing at a variety of sites, many of which are near or coincident with Rho-dependent termination sites [this paper; reviewed in Platt (1986)]. Because pausing is an essential component of termination (von Hippel et al., 1984), it seemed very plausible that Nus A caused pausing and changes in termination efficiency result from Nus A binding to a single site on the transcription complex. However, it is paradoxical that, in the absence of Nus A, increased pausing leads to increased termination (von Hippel et al., 1984), whereas Nus A increases pausing but decreases termination at Rho-dependent terminators (Lau & Roberts, 1983; Sigmund & Morgan, 1988). Therefore, it is equally possible that Nus A affects pausing and termination in vitro by unrelated mechanisms, perhaps by binding to different sites. The possibility that Nus A might bind functionally to more than one site is also suggested by the observations that Nus A may bind to RNA polymerase (Greenblatt & Li, 1981a), to RNA polymerase at more than one site (Schmidt & Chamberlin, 1984a), to Rho (Schmidt & Chamberlin, 1984b), to RNA at or near a sequence called box A (Tsugawa et al., 1985), and nonspecifically to RNA (Tsugawa et al., 1985).

Whether or not Nus A binds to a single site to affect both pausing and termination in vitro can be tested by measuring the magnitude of the effects of Nus A on pausing and termination at several Nus A concentrations. Although previous studies used nearly equimolar amounts of Nus A and RNA polymerase, the use of nearly equimolar amounts of RNA polymerase and Nus A does not ensure that the various effects observed are physiologically relevant, caused by the binding of Nus A to a single site, or quantitatively representative of the events that occur in vivo.

This paper addresses the following specific questions: (1) Do all concentrations of Nus A cause proportional increases in pausing at all pause sites, indicating that Nus A affects pausing by binding to a single site or to sites with conserved features? (2) Does Nus A cause effects on termination at the same concentrations that increase pausing, indicating that the effects of Nus A on pausing and termination are caused by binding to the same site? (3) What are the maximum magnitudes of the effects of Nus A on pausing and termination?

## Materials and Methods

*Analysis of in Vitro Transcripts.* In vitro transcription was performed as described in the companion paper (Sigmund & Morgan, 1988) except that single-round transcription was achieved by prewarming all reaction components except UTP at 37 °C for 10 min, at which time 10 $\mu$Ci of [$\alpha$-$^{32}$P]UTP at a final concentration of 40 $\mu$M and 10 $\mu$g/mL rifampicin were added (Lau et al., 1983). Reactions performed to measure pausing did not contain Rho. All reactions contained identical final salt and glycerol concentrations. Analysis of transcripts by gel electrophoresis and densitometry was performed as previously described (Sigmund & Morgan, 1988). Total yeast ribosomal proteins (TP80) were the generous gift of Dr. Jonathan Warner.

## Results and Discussion

Our accompanying paper (Sigmund & Morgan, 1988) shows that the effect of Nus A on termination at Rho-dependent and Rho-independent terminators is not qualitatively

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