Articles

Identification of a Warfarin-Sensitive Protein Component in a 200S Rat Liver Microsomal Fraction Catalyzing Vitamin K and Vitamin K 2,3-Epoxide Reduction†

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ABSTRACT: A partially purified, 200S submicrosomal fraction exhibiting thiol-dependent vitamin K_1 (vitamin K) and epoxide reductase activities has been isolated by partial solubilization of rat hepatic microsomes with sodium cholate and separation by centrifugation at 105000*g* into a discontinuous sucrose gradient. At pH 7.4, the rates of vitamin K and vitamin K 2,3-epoxide reduction per milligram of 200S fraction protein were equivalent and were 2.5–3.0 times faster than in microsomes. Reduction of vitamin K 2,3-epoxide occurred in a tightly coupled, two-step reaction initially to vitamin K and subsequently to vitamin K hydroquinone (vitamin KH_2). Incorporation of glycerol or sucrose and of sodium cholate into reaction mixtures equivalently affected the rates of both vitamin K and vitamin K 2,3-epoxide reduction, but in the case of epoxide metabolism, the ratios of vitamin KH_2/vitamin K were much lower, suggesting that the second reaction has been partially uncoupled from the first. A 14000–17000-dalton warfarin-sensitive protein (WSP) that participates in vitamin K and vitamin K 2,3-epoxide reduction in the 200S fraction was identified by incorporation of *N*-[^{3}H]ethylmaleimide ([^{3}H]NEM) into the catalytically active reduced form of one or more attached disulfides. Reduction of WSP with dithiothreitol was required for reaction with [^{3}H]NEM, and the substrates vitamin K and vitamin K 2,3-epoxide and the inhibitor warfarin all effectively blocked the reaction. 2-Mercaptoethanol could not substitute for dithiothreitol. On the basis of the data of these investigations, we conclude that warfarin inhibits reduction by dithiothreitol of the disulfide which in the reduced form undergoes reaction with NEM and is protected from NEM via the metabolism of vitamin K or vitamin K 2,3-epoxide. Since it is unlikely that the complex metabolism of vitamin K and vitamin K 2,3-epoxide could be catalyzed solely by WSP, it is probably part of a multienzyme system.

The enzyme-catalyzed reductions of vitamin K_1 (vitamin K)[1] to vitamin KH_2 and of vitamin K 2,3-epoxide to vitamin K are integral events in a cycle of vitamin K metabolism (Suttie, 1980). Vitamin KH_2 is a substrate for a γ-carboxylase of the cycle (Sadowski et al., 1976) which posttranslationally catalyzes Gla formation in the N-terminal regions of vitamin K dependent proteins (Hauschka et al., 1975; Friedman et al., 1979; Bell, 1980; Buchthal & Bell, 1980). During Gla formation, vitamin K 2,3-epoxide is formed as a product of the carboxylation reaction (Willingham & Matschiner, 1974; Larson et al., 1981) and undergoes sequential reduction to vitamin K and vitamin KH_2. These conversions therefore ensure that sufficient intracellular concentrations of vitamin KH_2 are maintained to support normal rates of Gla formation.

The thiol-dependent reductions of vitamin K and its epoxide are primarily catalyzed by the microsomal fraction of the liver (Zimmermann & Matschiner, 1974; Friedman & Shia, 1976). Warfarin and other 4-hydroxycoumarin drugs are potent inhibitors of these reactions in normal rats but are much less effective in warfarin-resistant rats (Zimmerman & Matschiner, 1974; Fasco et al., 1982). With the epoxide as substrate, both vitamin K and Vitamin KH_2 are formed in a "coupled" reaction where the initially formed metabolite, vitamin K, attains a steady-state rate while serving as a substrate for vitamin KH_2 formation (Fasco & Principe, 1982). Studies with model substrates have indicated that the sulfhydryl groups of a reduced protein disulfide bond could catalyze vitamin K 2,3-epoxide reduction (Silverman, 1981; Preusch & Suttie, 1983). In hepatic microsomes of rats, warfarin inhibited vitamin K 2,3-epoxide reduction by preventing dithiothreitol-mediated reduction of at least one protein disulfide bond (Fasco et al., 1983a). Vitamin K reduction and vitamin K 2,3-epoxide reduction were also both inhibitable by the sulfhydryl reagent NEM, after the dithiothreitol activation of one or more microsomal protein disulfide bonds. Vitamin K or vitamin K 2,3-epoxide added to dithiothreitol-treated microsomes prevented inhibition by subsequently added NEM of the metabolism of both vitamins (Lee & Fasco, 1984).

The complex interactions involved in vitamin K and vitamin K 2,3-epoxide reduction and their inhibition by warfarin will

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[1] Abbreviations: WSP, warfarin-sensitive protein; Gla, γ-carboxyglutamic acid; NEM, *N*-ethylmaleimide; vitamin K, vitamin K_1; vitamin KH_2, vitamin K hydroquinone; HPLC, high-performance liquid chromatography; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; % T, percentage total acrylamide monomer; % c, percentage *N,N'*-methylenebis(acrylamide); Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate·$2H_2O$; CHAPSO, 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate·$1.5H_2O$.

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be elucidated from studies with highly purified enzymes. Purification of the reductases has proved exceptionally difficult, however, because they are buried within the microsomal membrane (Carlisle & Suttie, 1980) and are readily denatured by many of the detergents required to achieve their solubilization (Siegfried, 1978; Hildebrandt et al., 1984).

We describe here a procedure for obtaining a partially purified, 200S submicrosomal fraction by cholate solubilization of hepatic microsomal membranes followed by centrifugation into a discontinuous sucrose gradient. Preparation of the vitamin K and vitamin K 2,3-epoxide reductases by this method has the advantages over other published procedures (Siegfried, 1978; Hildebrandt et al., 1984) that (i) the 200S particle is free of many of the protein and lipid components which can impede substrate, inhibitor, and cofactor transfer to the enzyme active sites, (ii) it contains the majority of the vitamin K and vitamin K 2,3-epoxide reductase activities initially present in microsomes, and (iii) it exhibits the same degree of coupled vitamin KH_2 formation as do microsomes with vitamin K 2,3-epoxide as the substrate. Using the 200S fraction, we have identified, by specific radiolabeling with [^{3}H]NEM, a 14000–17000-dalton WSP that participates in vitamin K and vitamin K 2,3-epoxide reduction.

EXPERIMENTAL PROCEDURES

Materials. Racemic warfarin, zwittergent 3-14, CHAPS, CHAPSO, and sodium cholate were purchased from Calbiochem-Behring, vitamin K was from Sigma, NEM (gold label) was from Aldrich, and emulgen 911 was from Kao Atlas (Tokyo, Japan). Vitamin K and vitamin K 2,3-epoxide were purified by HPLC and were dissolved at a concentration of 20 mg/mL in aqueous emulgen 911 (10% v/v) as described previously (Fasco & Kaminsky, 1980). Warfarin sodium salt was prepared by the method of West et al. (1961). Sep-Pak C_{18} cartridges (Waters Associates) were washed with methanol/water twice prior to use.

[^{3}H]NEM (45–50 Ci/mmol) in pentane was purchased from New England Nuclear. It was mixed with an aqueous solution of unlabeled NEM (80 mM) and the organic phase evaporated by a stream of nitrogen at 5 °C. Some NEM was lost under these conditions as determined by HPLC (see below), and unlabeled NEM was added to restore the concentration to 80 mM. The specific activities of the [^{3}H]NEM solutions used in each experiment and their percent radiochemical purity are cited in appropriate portions of the text.

The HPLC used was a Waters Associates Model 244 equipped with a recording integrator (Spectra Physics 4000) and a WISP autoinjector (Waters Associates). HPLC columns were radially compressed in a Z module (Waters Associates). Protein concentrations were determined by the method of Bradford (1976) using Bio-Rad reagents and bovine serum albumin as the standard. Cytochrome P-450 and P-420 concentrations were determined from difference spectra of their reduced vs. reduced–carbon monoxide complexes, and cytochrome b_5 concentrations were determined by oxidized vs. reduced difference spectra (Omura & Sato, 1964). Initial rates of vitamin K and vitamin K 2,3-epoxide metabolism were calculated by linear regression analysis.

Electrophoresis. SDS–PAGE was performed by a modification of the Laemmli (1970) method using reagents purchased from Bio-Rad. The resolving gel (11.5 × 14 × 0.15 cm) was 8.5 or 10% T, 3% c in 0.375 M Tris-HCl, and 0.1% SDS buffer, pH 8.8. The running buffer was 0.025 M Tris-HCl, 0.192 M glycine, and 0.1% SDS, pH 8.3. In some instances, the resolving gel was preelectrophoresed overnight at 10–15 °C in the running buffer. A stacking gel (0.8 × 14 × 0.15 cm) of 3.5% T, 2.6% c in 0.125 M Tris-HCl, and 0.1% SDS buffer, pH 6.8, was applied, and separation of the proteins was achieved at a constant current of 10 mA/gel for stacking and 20 mA/gel thereafter. Bromophenol blue was in the front marker. Proteins were visualized by staining with Coomassie Brilliant Blue R-250.

For determination of [^{3}H]NEM incorporation into protein, stained or unstained gel lanes containing the resolved proteins were partially frozen on dry ice and cut into 2-mm slices with a Model 190 gel slicer (Bio-Rad). Each slice was digested in a scintillation vial with 0.6 mL of 30% hydrogen peroxide at 80 °C overnight. Ready-Solv HP/b (10 mL, Beckman) was added with vortex mixing and the extent of [^{3}H]NEM incorporation determined by scintillation counting.

HPLC Separations. For the separation of NEM from its adducts with 2-mercaptoethanol and dithiothreitol, the column used was a Radial-Pak μBondapak C_{18} (Waters Associates). The solvent was water/acetonitrile/2-propanol (65/32/3) delivered at a flow rate of 1 mL/min. Detection was at 254 nm.

The column used for separation of the vitamins was a Waters Associates Radial-Pak C_{18} (5-mm internal diameter, 10-μm particle size) equilibrated in acetonitrile/2-propanol (9/1). The flow rate was 1 mL/min, and detection was at 254 nm. The elution times of vitamin KH_2, vitamin K 2,3-epoxide, and vitamin K were approximately 340, 480, and 790 s, respectively.

Concentrations of NEM and each vitamin were determined by comparisons of their integrated areas with those of external standards.

Preparation of the Submicrosomal Particle Fraction. Male Wistar rats (250 ± 20 g) received water and food ad libitum and were acclimatized to a 12-h on–off light cycle. The rats were fasted for 12 h before carbon dioxide induced anesthesia. The livers were perfused in situ with physiological saline and the microsomes isolated by differential centrifugation as previously described (Fasco & Principe, 1982). All subsequent operations were performed at 0–5 °C. The microsomal pellets were resuspended with a Wheaton homogenizer in sufficient 0.02 M Tris-HCl and 0.15 M KCl buffer, pH 7.4, to produce a final protein concentration of 20 mg/mL. Aliquots of this microsomal suspension were frozen at −80 °C until use.

The frozen microsomes were partially thawed by gentle warming at 25 °C and were diluted to a concentration of 10 mg of protein/mL with cold 0.2 M Tris-HCl and 0.15 M KCl buffer, pH 7.4. Aqueous sodium cholate (10%) was added slowly with gentle stirring to a final concentration of 0.5%. A 10-mL portion of the partially solubilized microsomes was immediately layered over 5 mL of 20% sucrose in 0.2 M Tris-HCl and 0.15 M KCl buffer, pH 7.4, atop 5 mL of 30% sucrose in the same buffer, and the mixture was centrifuged at 105000g for 1 h. Aliquots of the various fractions obtained after the centrifugation (see Results) were assayed for vitamin K and vitamin K 2,3-epoxide reductase activities as described below.

Determination of the Sedimentation Coefficient. The sedimentation coefficient of the sucrose fraction containing the majority of the vitamin K and vitamin K 2,3-epoxide reductase activities was determined at 4 °C with a Beckman SW 41 Ti rotor using a linear 10–30% sucrose gradient. The run time was 1 h at 40000 rpm. Location of the reductase activities in the sucrose gradient was determined by enzymic assay (see below), and the center of the band was used as the measure of the migration distance. Calculation of the sedimentation coefficient was from a $s\omega^2t$/distance standard curve supplied by Beckman (Applications Data Sheet DS-528).

Assay of Vitamin K and Vitamin K 2,3-Epoxide Reduction. (*A*) *In Microsomes.* Reaction mixtures contained 6 mg of microsomal protein in 1.9 mL of 0.2 M Tris-HCl and 0.15 M KCl buffer, pH 7.4. Vitamin K or vitamin K 2,3-epoxide (0.01 mL) was added, and the mixture was incubated for 1 min at 25 °C. Reaction was initiated by the addition of 0.1 mL of dithiothreitol (0.1 M) and was terminated after various incubation times at 25 °C with 4 mL of 2-propanol/hexane (1/1). The vitamins were extracted by a brief vortex mixing, and following a 15-s centrifugation at low speed, a 1.5-mL aliquot of the solvent phase was removed and evaporated to dryness at 35 °C under a stream of oxygen-free nitrogen. As essentially constant, rapid rate of solvent evaporation was maintained by continually lowering the nitrogen stream while rotating it around the interior walls of the tube. This technique prevents reaction between vitamin K or its epoxide with any contaminating dithiothreitol (Fasco & Principe, 1980; Fasco et al., 1983b). The residue was dissolved in 0.2 mL of 2-propanol, and a 0.02-mL aliquot was immediately assayed by HPLC.

(*B*) *In Isolated Fractions.* The method was essentially that used for microsomes except that 0.6 mL of the appropriate fraction was added to 1.3 mL of the 0.2 M Tris-HCl and 0.15 M KCl buffer, pH 7.4. In other experiments, dilution was with 0.2 M Tris-HCl and 0.15 M KCl buffer, pH 8.5, containing sufficient glycerol or sucrose and sodium cholate to produce final concentrations of 20% glycerol (v/v) or 20% sucrose and 0.7% sodium cholate. Vitamin K or vitamin K 2,3-epoxide was added in 1% emulgen 911, and the reaction mixtures were incubated for 1 min at 25 °C before initiation of the reaction with 0.1 mL of dithiothreitol (0.1 M). In the cases where reaction mixtures contained 20% glycerol or sucrose, an additional 1 mL of water was added after the reaction was terminated with 2-propanol/hexane (4 mL) to reduce extraction of these components into the solvent phase. Other procedures in the extraction and HPLC assay of the vitamin components were as described for the microsomal system.

Warfarin and NEM Inhibition Studies. An aliquot of the 200S fraction (1.2 mL) was diluted with 2.6 mL of 0.2 M Tris-HCl and 0.15 M KCl buffer, pH 7.4. Sodium warfarin (2 μM final concentration) was added in 0.01 mL of water and the mixture incubated at 25 °C for 1 min. It was then passed through a Sep-Pak C_{18} cartridge (Waters Associates) at a flow rate of 4–8 mL/min. The Sep-Pak run-through fractions from three or four identically treated samples were pooled for subsequent determination of the effect of warfarin on the fraction. In the case of NEM, dilution of the 200S fraction was the same as described for warfarin, but just before incubation at 25 °C, 0.02 mL of dithiothreitol (0.1 M) was added for reduction of any sensitive protein disulfide bonds. Water (0.02 mL) was added for studies of NEM effects on oxidized proteins. At 1 min of incubation, 0.1 mL of NEM (80 mM in H_2O) was added, and after an additional 1 min, the mixtures were passed through a Sep-Pak cartridge. The eluates from multiple Sep-Paks were pooled for determination of the effects of NEM on the oxidized 200S fraction and on the dithiothreitol-reduced fraction. Under these conditions, only trace quantities of unbound NEM and any of the adducts formed from reaction with dithiothreitol were eluted with the 200S fraction.

[^{3}H]NEM Labeling Studies. A 1.2-mL aliquot of the 200S fraction (3.5–4 mg of protein/mL) was diluted with 2.6 mL of 0.2 M Tris-HCl and 0.15 M NaCl buffer, pH 7.4, and was incubated at 25 °C for 1 min. NEM (80 mM, 0.2 mL) was added, and after an additional 4-min incubation, the unreacted NEM was removed by passage of the mixture through a Sep-Pak C_{18} cartridge (Waters Associates). Residual enzyme was recovered by forcing air through the cartridge. The total eluate was collected in a vial cooled in ice. When larger samples were required, the eluates from identically treated samples were combined after each had cooled to ice temperature.

In studies to determine the extent of overall [^{3}H]NEM incorporation into proteins of the 200S fraction, conditions were as described above except that no unlabeled NEM was added. A 1.9-mL aliquot of the Sep-Pak eluate was incubated at 25 °C for 1 min, and [^{3}H]NEM (80 mM, 0.012 mL) was added with vortex mixing. After 1 additional min, the mixture was passed through a second Sep-Pak to remove any unreacted [^{3}H]NEM, and the eluate was analyzed for [^{3}H]NEM incorporation into protein by SDS–PAGE.

For determination of the effects of various combinations of 2-mercaptoethanol, dithiothreitol, vitamin K, vitamin K 2,3-epoxide, and warfarin on the incorporation of [^{3}H]NEM into protein, conditions were similar to those just described with the following modifications: (i) aliquots (0.95 mL) of the 200S fraction which had been treated with 4 mM NEM (unlabeled) and passed through a Sep-Pak were used; (ii) emulgen 911 was included in all reaction mixtures at the same concentration; and (iii) 2-mercaptoethanol (0.1 M, 0.005 mL), dithiothreitol (0.1 M, 0.005 mL), vitamin K (4.4 mM in 1% emulgen 911, 0.005 mL)/dithiothreitol (0.1 M, 0.005 mL), vitamin K 2,3-epoxide (4.4 mM in 1% emulgen 911, 0.005 mL)/dithiothreitol (0.1 M, 0.005 mL), or warfarin (0.01 M, 0.005 mL)/dithiothreitol (0.1 M, 0.005 mL) was (were) added in the order specified just before the mixtures were incubated at 25 °C. After 1 min, [^{3}H]NEM (80 mM, 0.012 or 0.025 mL) was added with vortex mixing, and after 20 s, the mixtures were chromatographed on the Sep-Paks, the eluates being collected in tubes cooled in ice. SDS (2% in water, 0.1 mL) was added, and the mixtures were stored at −80 °C until assayed for [^{3}H]NEM incorporation into protein by the SDS–PAGE method described.

In related experiments designed to determine the effects of these treatments on vitamin K or vitamin K 2,3-epoxide reductase activity, unlabeled NEM was substituted for the ^{3}H-labeled compound. The Sep-Pak eluates from 0.95-mL incubations were diluted with dithiothreitol (0.1 M, 0.05 mL) and incubated at 25 °C for 1 min. Vitamin K or vitamin K 2,3-epoxide (4.4 mM in 1% emulgen 911, 0.005 mL) was added, and after 10 additional min at 25 °C, the reactions were terminated by the addition of 1 mL of water and 4 mL of hexane/2-propane (1/1). The extents of vitamin metabolism were determined by HPLC as described.

RESULTS

In these investigations, the extent of coupling that occurred with vitamin K 2,3-epoxide as the substrate was arbitrarily defined from the ratio of the vitamin KH_2/vitamin K metabolite concentrations after 10 min of reaction, providing that the initial rates of vitamin K and vitamin K 2,3-epoxide metabolism remained equivalent. Partially purified reductase preparations that produced ratios equivalent to or greater than the microsomal ratio of 2–2.5 were deemed to be well coupled, whereas those less than 2 were deemed to be partially "uncoupled". Isolation of the vitamin K and vitamin K 2,3-epoxide reductases by the method described below was highly reproducible, and numerous preparations have been made over the past year without any appreciable variations in reductase and coupled activities. The preparations have been stored at

Table I: Distribution of the Vitamin K and Vitamin K 2,3-Epoxide Reduction Activities in a Discontinuous Sucrose Gradient[a]

source of enzyme	volume (mL)	protein concn (mg/mL)	total protein (mg)	reductase act.[a,b] [nmol $(10\ \text{min})^{-1}$ (mg of protein)$^{-1}$]		$[KH_2]/[K]$[c] ratio	total reductase act.[d]		% total microsomal act.	
				K	KO		K	KO	K	KO
microsomes	10	10	100	1.1	1.2	2.3	110	122	100	100
fraction 1	2.9	3.2	9.3	ND[e]	ND				0	0
fraction 2	7.4	7.4	54.8	ND	0.1	ND		3.5		2.9
fraction 3	2.0	4.3	8.6	1.0	1.1	1.3	8.6	9.3	7.8	7.9
fraction 4	7.1	4.0	28.4	2.9	3.3	2.1	82.8	92.3	75.3	75.7
fraction 5	0.7	4.1	2.9	1.9	2.3	1.7	5.6	6.5	5.1	5.3
pellet	1.0	1.5	1.5[f]	0.1	0.2	ND	0.2	0.3	0.1	0.3
fraction totals	20.1		105.5				97.2	111.9	88.3	92.1

[a] Enzymic assays were performed as described under Experimental Procedures. [b] The substrates were vitamin K (K) and vitamin K 2,3-epoxide (KO). The rates are vitamin KH_2 formed from vitamin K and the sum of vitamins KH_2 and K formed from vitamin KO. [c] The vitamin KH_2 and vitamin K concentrations were determined at 10 min of reaction with vitamin K 2,3-epoxide as the substrate. [d] The total reductase activity equals the K or KO reductase activity times the total protein. [e] Not detectable. [f] The pellet was resuspended by homogenization in 0.2 M Tris-HCl and 0.15 M KCl buffer, pH 7.4, containing 1% sodium cholate and 20% sucrose for protein determination and assay.

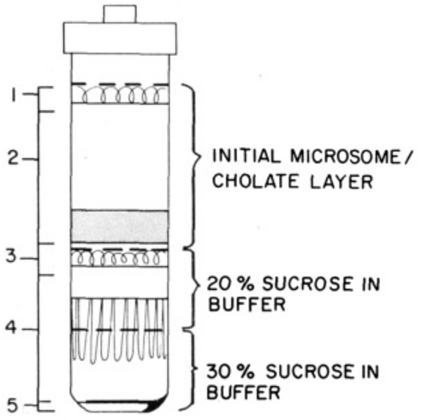

FIGURE 1: Representation of the visual banding pattern obtained following partial solubilization of hepatic microsomes with 0.5% sodium cholate and centrifugation at 105000*g* over a 20–30% sucrose gradient. Conditions were as described under Experimental Procedures. A detailed description of the appearance of the various regions is included in the text.

−80 °C for at least 6 months without detectable loss of enzymic activity.

A representation of the visible banding pattern obtained after partial solubilization of the microsomes with 0.5% sodium cholate and centrifugation at 105000*g* for 1 h over a 20%/30% discontinuous sucrose gradient is presented in Figure 1. The distribution of the vitamin K and vitamin K 2,3-epoxide reductase activities among the five fractions illustrated in Figure 1 is presented in Table I. The vast majority of the vitamin K and vitamin K 2,3-epoxide reductase activities were associated with fraction 4. This fraction also exhibited good coupled vitamin KH_2 formation with vitamin K 2,3-epoxide as the substrate, and therefore, it was used as the source of enzyme for vitamin K and vitamin K 2,3-epoxide reduction in these investigations.

Approximately 20–25% of the microsomal cytochrome P-450 and 45–50% of the microsomal cytochrome b_5 were not solubilized by the cholate and cosedimented with fraction 4. The UV maximum of fraction 4 was at 260 nm, suggestive of the presence of nucleic acid and/or flavoprotein.

The specific activity of fraction 4 with respect to both vitamin K and vitamin K 2,3-epoxide reduction was normally 2.5–3.0 times higher than in microsomes when assayed at pH 7.4 and in the absence of detergent. As described in detail below, however, the rates of both vitamin K and vitamin K 2,3-epoxide reduction in fraction 4 could be markedly enhanced by incorporation of sodium cholate, glycerol, or sucrose and increasing the pH of reaction mixtures. In contrast, microsomes containing 0.5% sodium cholate at pH 7.4 exhibited a 50% loss of activity which was at least partially restored by removal of the detergent during centrifugation into the sucrose. Therefore, the specific activity and percent recovery values in Table I are only apparent and are presented primarily to indicate the distribution of reductase activities among the various fractions. Centrifugation of fraction 4 through a 10–30% sucrose gradient produced a single, diffuse band which contained more than 90% of the vitamin K and vitamin K 2,3-epoxide reductase activities initially applied. Its sedimentation coefficient measured to the center of the band was 200 S, and for the purpose of clarity, the term 200S fraction will be used hereafter to describe fraction 4.

Initial studies with the 200S fraction undertaken to optimize the conditions for vitamin K and vitamin K 2,3-epoxide metabolism demonstrated that the rates of their metabolism could be dramatically altered depending on the pH and on the concentrations of detergent and/or stabilizing medium such as glycerol or sucrose present. In each of the various conditions examined, however, the rates of vitamin K and vitamin K 2,3-epoxide metabolism were affected equivalently, but the extent of vitamin KH_2–vitamin K coupling during epoxide metabolism was markedly diminished. Since such data provide insight into the factors that regulate coupling of these metabolites, detailed investigations of vitamin K and vitamin K 2,3-epoxide metabolism under various conditions were undertaken.

Relative to the profiles of vitamin K and vitamin K 2,3-epoxide reduction at pH 7.4, increasing the pH to 8.5 and incorporation of 0.7% sodium cholate and 20% glycerol or sucrose into the reaction mixtures markedly enhanced the initial metabolism rates (Figure 2), the changes being equivalent for each vitamin substrate (see legend). Over the course of epoxide metabolism at pH 7.4 (Figure 2B insert), vitamin K was the only metabolite detected initially, and it reached a steady-state concentration within 1–2.5 min of reaction which was maintained over the range of time investigated. Following an initial lag in its formation, the concentration of vitamin KH_2 increased steadily until, at 10 min of reaction, its concentration was nearly 3 times that of vitamin K. In the presence of 0.7% sodium cholate and 20% glycerol at pH 8.5, the steady-state concentration of vitamin K was approximately 3-fold higher,

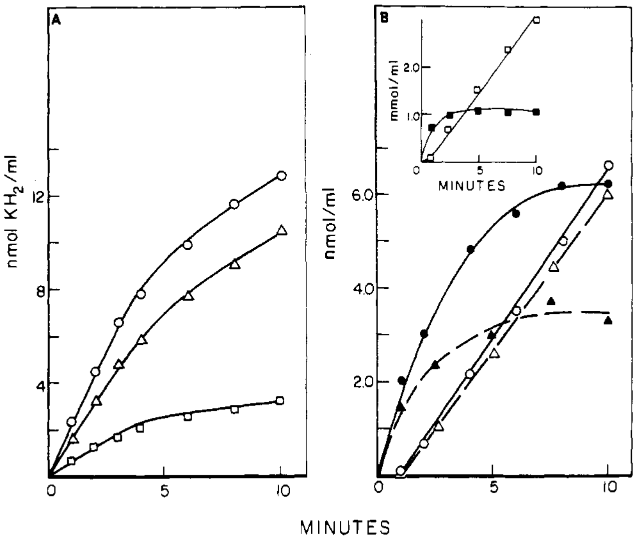

FIGURE 2: Effects of different incubation conditions on the vitamin K to vitamin KH_2 (A) and the vitamin K 2,3-epoxide to vitamin K and vitamin KH_2 (B) conversions by the 200S fraction as a function of time. Reaction mixtures were 2 mL and were prepared as described under Experimental Procedures for the assay of vitamin reduction in isolated fractions. The initial substrate concentrations were 0.02 mM. The conditions for the reactions were (□) pH 7.4, (△) pH 8.5, 0.7% sodium cholate and 20% glycerol, and (○) pH 8.5, 0.7% sodium cholate and 20% sucrose. In panel B, the open symbols are vitamin KH_2 concentrations, and the closed symbols are vitamin K concentrations. The initial rates of vitamin KH_2 formation in (A) were respectively 0.48, 1.23, and 1.64 nmol min^{-1} (mg of protein)$^{-1}$, and the rates of vitamin K plus vitamin KH_2 formation from the epoxide in (B) were 0.58, 1.20, and 1.44 nmol min^{-1} (mg of protein)$^{-1}$.

but the vitamin KH_2/vitamin K ratios were consistently lower throughout the course of the reaction, and at 10 min, the ratio was 1.7. Substitution of 20% sucrose for glycerol increased the steady-state vitamin K concentration nearly 6-fold over that obtained at pH 7.4, but as with glycerol, the vitamin KH_2 formation was slowed, and at 10 min of reaction, the vitamin KH_2/vitamin K ratio was reduced to 1.

To assess more definitively the role of sodium cholate in the reaction, the effects of its concentration on vitamin K and vitamin K 2,3-epoxide metabolism at pH 7.4 in 20% sucrose were determined (panels A and B, respectively, of Figure 3). At 0.1%, sodium cholate inhibited the metabolism of both vitamins. At higher concentrations, however, the rates of metabolism of each vitamin increased steadily to maximum values between 0.4% and 0.6% sodium cholate and then declined. Within the estimated error of the experiment, the rates of vitamin K and vitamin K 2,3-epoxide metabolism were equivalent at each sodium cholate concentration investigated. However, the relative proportions of vitamin KH_2 and vitamin K formed during epoxide metabolism changed markedly with the sodium cholate concentration. At 0.8% sodium cholate, no vitamin KH_2 was detectable although the rate of epoxide metabolism was equivalent to that in the absence of sodium cholate where the vitamin KH_2 concentration was 2-fold higher than the vitamin K concentration. Moreover, vitamin KH_2 formation during epoxide metabolism was optimal at 0.3–0.4% sodium cholate, whereas in the case where vitamin K was the substrate (Figure 3A), vitamin KH_2 formation was maximal at higher concentrations.

The effects of warfarin and NEM on vitamin K 2,3-epoxide metabolism as a function of time are presented in Figure 4. Preliminary experiments had demonstrated that 2 μM warfarin or treatment of the 200S fraction with 0.5 mM dithiothreitol prior to exposure to 2 mM NEM for 1 min produced approximately a 50–80% inhibition of epoxide reduction. The

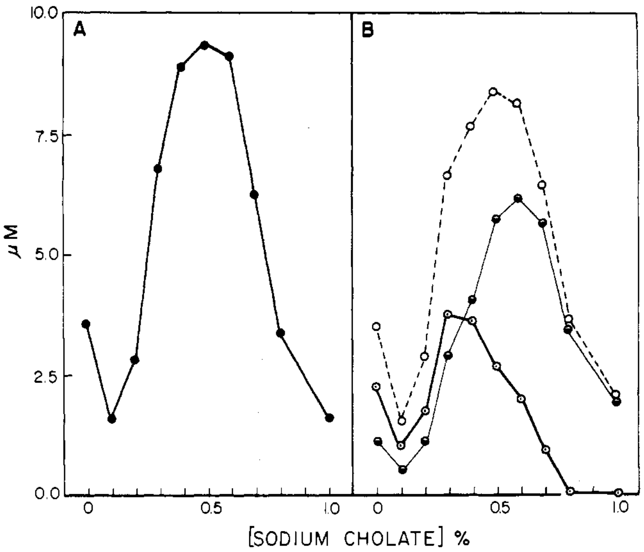

FIGURE 3: Effects of sodium cholate concentrations on (A) the conversion of vitamin K to vitamin KH_2 (●) and (B) the conversion of vitamin K 2,3-epoxide to vitamin K (⊖) and vitamin KH_2 (⊙). The open circles in panel B are the sums of the vitamin K and vitamin KH_2 concentrations. Reaction mixtures (2 mL) contained 20% sucrose and were prepared as described under Experimental Procedures for the assay of vitamin K and vitamin K 2,3-epoxide reduction in isolated fractions. The pH was 7.4.

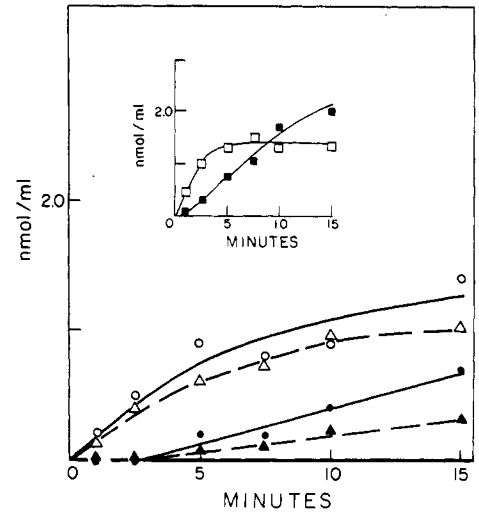

FIGURE 4: Effects of NEM and warfarin on the individual vitamin K and vitamin KH_2 concentrations formed by the 200S particle fraction as a function of time. The conditions were as described for Warfarin and NEM Inhibition Studies under Experimental Procedures. The conditions are (□) no inhibitor, (○) 0.5 mM dithiothreitol and 2 mM NEM, and (△) 2 μM warfarin. Vitamin K and vitamin KH_2 concentrations are respectively represented by open and closed symbols.

profiles of metabolites formed following either 4-min incubation of the 200S fraction at 25 °C or 4-min incubation with 2 mM NEM followed by Sep-Pak chromatography to remove unbound inhibitor and any dithiothreitol reaction products were indistinguishable from one another. However, the Sep-Pak chromatography slightly uncoupled vitamin KH_2 formation (cf. Figures 2 and 4). The extent of warfarin inhibition of epoxide metabolism was essentially the same irrespective of whether or not the samples were chromatographed through a Sep-Pak. As shown in Figure 4, both warfarin and NEM bound to the sulfhydryl groups of one or more target disulfide bonds slowed the initial rate of vitamin K formation and had a marked inhibitory effect on vitamin KH_2 formation. Indeed, when the slightly different extents of inhibition are considered,

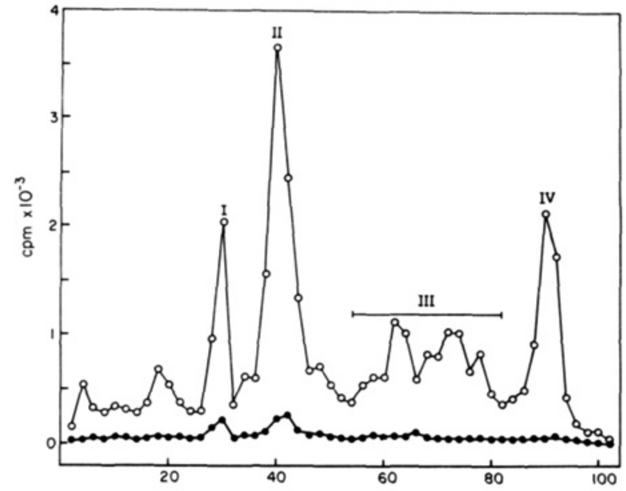

FIGURE 5: Relative incorporation of [^{3}H]NEM into (○) untreated 200S proteins and (●) 200S proteins previously exposed to unlabeled NEM (2 mM) for 4 min. Samples containing untreated 200S proteins (1 mg/mL) were diluted and chromatographed on C_{18} Sep-Pak cartridges in the same way as were the treated samples. A sample (0.95 mL) of each was incubated at 25 °C for 1 min and exposed to [^{3}H]NEM (1 mM, 164 μCi/μmol, 79% isotopically pure) for 1 min. Excess [^{3}H]NEM was removed by Sep-Pak C_{18} chromatography. Samples (0.07 mL) contained 0.2% SDS, and 1% 2-mercaptoethanol prior to separation by SDS–PAGE. The resolving gel composition was 8.5% T and 3% c. The proteins were visualized with Coomassie Brilliant Blue R-250 and assayed by scintillation counting as described under Experimental Procedures.

both warfarin and NEM produced virtually the same profile of inhibition, suggesting that both agents affect the same site.

Attempts to further fractionate the reductases in the 200S fraction resulted in a marked loss of unrecoverable enzymic activity. Covalent modification studies with [^{3}H]NEM were therefore undertaken to identify the protein component(s) containing the dithiothreitol-reducible and NEM-sensitive disulfide. The profile of [^{3}H]NEM-labeled proteins obtained by exposing the 200S particle fraction to 1 mM [^{3}H]NEM for 1 min at 25 °C and, following removal of excess labeling reagent by Sep-Pak chromatography, separating the component proteins by SDS–PAGE under reducing conditions is shown in Figure 5. Boiling the reduced samples had no detectable effect on the distribution of [^{3}H]NEM. Four groups of [^{3}H]NEM-incorporating proteins (I–IV) were arbitrarily assigned on the basis of this incorporation and will be referred to by this designation throughout. To reduce the high levels of nontarget incorporation [^{3}H]NEM into the 200S fraction proteins, the possibility of first exposing them to unlabeled NEM was investigated. The resultant loss of nontarget incorporation produced by initial exposure to 2 mM unlabeled NEM for 4 min at 25 °C is shown in Figure 5. In subsequent experiments, the concentration of unlabeled NEM used to block the nontarget groups in the 200S fraction was increased to 4 mM. This concentration reduced the background still further and, importantly, had no detectable effect on the rates of vitamin K and vitamin K 2,3-epoxide reduction.

The effects of 0.5 mM 2-mercaptoethanol, 0.5 mM dithiothreitol, and 0.05 mM warfarin/0.5 mM dithiothreitol on the subsequent incorporation of [^{3}H]NEM (2 mM) into proteins of the 200S fraction previously treated with 4 mM unlabeled NEM for 4 min are illustrated in Figure 6. Relative to the pattern of [^{3}H]NEM-labeled proteins obtained with 2-mercaptoethanol, dithiothreitol promoted the incorporation of [^{3}H]NEM principally in the region of peak group IV. Warfarin at a concentration of 50 μM completely blocked dithiothreitol-mediated incorporation of [^{3}H]NEM into the peak IV region. Similar results were obtained when reaction mixtures contained dithiothreitol and vitamin K or vitamin K 2,3-epoxide added prior to the [^{3}H]NEM (data not shown). The protein composition of the 200S fraction visualized by staining with Coomassie Brilliant Blue is presented in the insert of Figure 6. Proteins of known molecular weight and electrophoresed in the same gel are included in the lane on the right. The brace indicates the region containing peak group IV which is 14000–17000 daltons. Essentially the same profiles were obtained whether the [^{3}H]NEM-labeled proteins were separated by SDS–PAGE under reducing (1% 2-mercaptoethanol) or nonreducing conditions, suggesting that any components of peak group IV containing dithiothreitol-reducible disulfide bonds are composed of one or more single-chain polypeptides that are not covalently attached via interchain disulfides to any other protein.

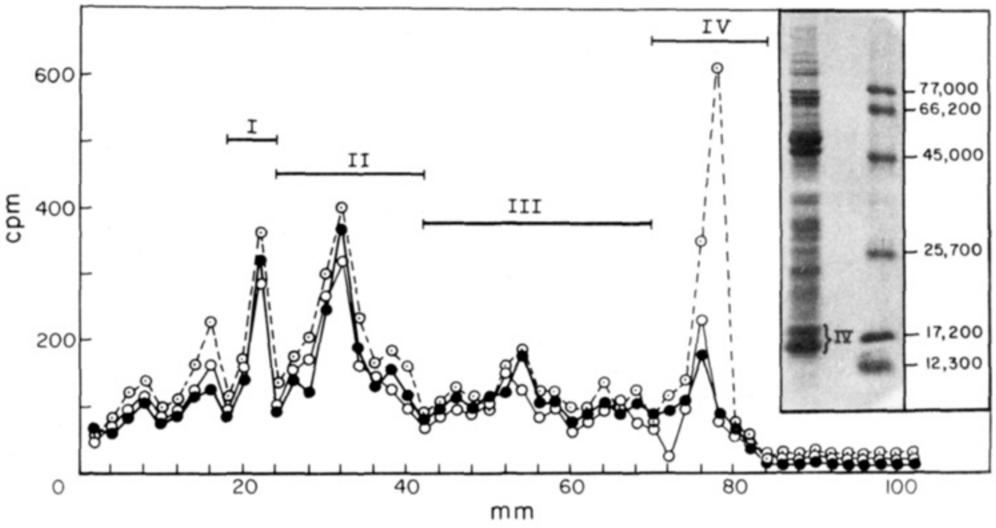

FIGURE 6: Effects of treatment of the 200S fraction with (●) 2-mercaptoethanol (0.5 mM), (⊙) dithiothreitol (0.5 mM), and (○) warfarin (0.05 mM)/dithiothreitol (0.5 mM) on the subsequent incorporation of [^{3}H]NEM (2 mM, 300 μCi/μmol, 98% isotopically pure). The 200S proteins (1 mg/mL) were initially treated with unlabeled NEM (4 mM) for 4 min at 25 °C, and the excess reagent was removed by Sep-Pak C_{18} chromatography. Other conditions were as described in Figure 5 except that exposure to the [^{3}H]NEM was for 20 s, and the resolving gel composition was 10% T/3% c. It was preelectrophoresed overnight prior to the pouring of the stacking gel. Insert: left lane, profile of the 200S proteins separated by SDS–PAGE under reducing conditions and visualized with Coomassie Brilliant Blue R-250; right lane, protein standards run in the same gel.

DISCUSSION

Of the many detergents tested during our initial investigations, including sodium deoxycholate, emulgen 911, emulgen 913, CHAPS, CHAPSO, and zwittergent 3-14, 0.5% sodium cholate was found to be most effective in solubilizing many microsomal proteins such as cytochrome P-450 while at the same time preserving most of the vitamin K and vitamin K 2,3-epoxide reductase activities, including coupled vitamin KH_2 formation. At this cholate concentration, however, virtually all of both reductase activities partitioned above and below the 20–30% sucrose interface during centrifugation at 105000g for 1 h, demonstrating, in agreement with Hildebrandt et al. (1984), that they were not completely disaggregated by cholate. Under the conditions used for enzymic assay at pH 7.4 and in the absence of any detergent not already present, the 200S fraction was slightly opalescent. Vitamin K and epoxide reduction rates with the 200S fraction were 2–3-fold faster than with microsomes per milligram of protein, and vitamin KH_2 formation during epoxide metabolism occurred with a vitamin KH_2/vitamin K coupling ratio comparable to that of the microsomal system. Treatment of the 200S fraction with Tris-HCl buffer (either pH 7.4 or 8.5) and 0.7% cholate/20% sucrose produced a mixture that was visually clear and exhibited excellent vitamin K and vitamin K 2,3-epoxide reductase activities, but the vitamin KH_2/vitamin K coupling ratio was dramatically altered under these conditions.

There is currently conflicting evidence as to whether vitamin K is an inhibitor of vitamin K 2,3-epoxide metabolism and whether these substrates are metabolized at one or more catalytic sites (Preusch & Suttie, 1984; Lee & Fasco, 1984). Data obtained with the partially purified, 200S fraction support our previous microsomal data indicating that the metabolism of these two vitamins occur at separate, independent sites (Lee & Fasco, 1984). Vitamin K 2,3-epoxide metabolism at pH 8.5 in the presence of 20% sucrose and 0.7% cholate was sufficiently rapid that more than half of the initially added substrate was metabolized during this time. Thus, within 5 and 10 min of reaction, the vitamin K metabolite and epoxide substrate concentrations were approaching equal values without any concomitant deviation in the rate of substrate disappearance, indicating that no antagonism exists between these two vitamins. Moreover, it was consistently observed that the vitamin KH_2/vitamin K coupling ratio was markedly lowered by a variety of perturbations of the 200S fraction, but under identical conditions, the rates of vitamin KH_2 formation when vitamin K was the substrate were affected equivalently to those of epoxide reduction. Presumably, the sites of vitamin K 2,3-epoxide and vitamin K metabolism have been spatially altered relative to one another under these conditions, and the vitamin K formed from the epoxide must "travel" a greater distance to reach its site of metabolism. Higher vitamin K and lower vitamin KH_2 concentrations would be expected to occur in this situation because more metabolically formed vitamin K would be lost due to diffusion, thus slowing the second reaction. When vitamin K is used as the substrate, the element of diffusion is removed, and the reaction proceeds at an apparently normal rate.

Preliminary attempts to further purify the vitamin K and epoxide reductases in the 200S fraction were unsuccessful because of the strong attractions between the component proteins and their sensitivity to the detergents and conditions necessary to achieve their separation. A method of identifying at least one of the enzymes involved in the reactions was devised by specifically and covalently radiolabeling it with [^{3}H]NEM. From the data presented in Figure 6, only one of the peak groups in the 200S fraction contained one or more disulfide bonds that reacted significantly with [^{3}H]NEM after reduction with dithiothreitol and none after reduction with 2-mercaptoethanol. Moreover, within the estimated error of the experiment, [^{3}H]NEM incorporation into the sulfhydryl groups of all of the dithiothreitol-reducible disulfides in the peak group IV was completely blocked by the inhibitor warfarin and the substrate vitamin K or vitamin K 2,3-epoxide.

Data from enzymic studies of vitamin K and vitamin K 2,3-epoxide antagonism of inhibition by NEM have indicated that both vitamins act on a single, common disulfide involved in their metabolism (Lee & Fasco, 1984). Incubation with either vitamin almost completely blocked [^{3}H]NEM incorporation into the reduced form of the peak group IV component, supporting the concept that a single disulfide is involved. Moreover, warfarin completely blocked [^{3}H]NEM incorporation into the same 200S fraction component, and on the basis of the data in Figure 4, warfarin and NEM affect the same disulfide, albeit by different mechanisms. For these reasons, the [^{3}H]NEM-labeled component of peak group IV is probably a single polypeptide chain containing one disulfide-containing component which exhibits the properties just described.

The ability to differentiate the sulfhydryl groups of the target disulfide that participates in vitamin K and vitamin K 2,3-epoxide metabolism from the sulfhydryl groups of potentially numerous other disulfides or other nontarget NEM reactive groups present in the complex protein–lipid milieu of the microsomal membrane required that small differences in [^{3}H]NEM incorporation into the component proteins be accurately quantified in a number of variously treated samples. Evidence for the precision and reproducibility of the method developed can be derived from the data in Figure 6. The levels of radiolabel incorporated into the peak groups I–III were remarkably similar (considering the fact that the reaction mixtures initially contained as much as 120×10^6 cpm of [^{3}H]NEM/mL) and serve as internal controls for the enhanced incorporation of [^{3}H]NEM in peak group IV in the presence of dithiothreitol.

The identification of a 14000–17000-dalton WSP is the first evidence that vitamin K metabolism and vitamin K 2,3-epoxide metabolism are catalyzed by a multienzyme system. Because of the complexity of vitamin K and particularly vitamin K 2,3-epoxide reduction, it is unlikely that a single species of this relatively low molecular weight could catalyze these reactions. In addition to the disulfide component identified here, WSP would have to contain individual binding sites and catalytic groups for vitamin K and vitamin K 2,3-epoxide reduction as well as a warfarin binding site (Fasco et al., 1983). Much additional experimentation is thus required to ascertain how many other subunits are involved in the reactions, their molecular weights and functional catalytic groups, and their roles in vitamin K and vitamin K 2,3-epoxide metabolism.

On the basis of the available enzymic and [^{3}H]NEM data, it is probable that the target disulfide described here is not composed of the sulfhydryl groups from two cysteine residues. They are normally of low reactivity and would not be expected to react in concert as apparently is the case for the sulfhydryl groups of this catalytically active disulfide. A disulfide-containing compound analogous to lipoic acid would be a possible candidate—it is reducible by other dithiols, and bound to protein it has sufficient chain length to function in the transfer of reducing equivalents from one protein subunit to another as has been postulated to occur during vitamin K and vitamin K 2,3-epoxide metabolism (Lee & Fasco, 1984). Studies in this laboratory are currently in progress to purify the enzyme component of peak group IV in a biologically active state and to ascertain the structure of this extremely important disulfide moiety.

Registry No. Vitamin K_1 reductase, 9032-20-6; vitamin K_1 2,3-epoxide reductase, 55963-40-1.

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Biological Activity and Conformational Isomerism in Position 9 Analogues of the Des-1-tryptophan,3-β-cyclohexylalanine-α-factor from *Saccharomyces cerevisiae*[†]

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ABSTRACT: Analogues of the des-1-tryptophan,3-β-cyclohexylalanine-α-factor of *Saccharomyces cerevisiae*, where the glycyl residue of position 9 was replaced by D-Ala, L-Ala, D-Leu, and L-Leu, were synthesized and evaluated by morphogenesis assays and circular dichroism spectroscopy. Synthesis was accomplished in solution phase with mixed anhydrides and *p*-nitrophenyl active esters as the coupling agents. All crude dodecapeptides were purified to >98% homogeneity by preparative high-performance liquid chromatography on a reversed-phase column. The Gly^9, D-Ala^9, and D-Leu^9 analogues elicited morphogenic alterations in *MATa* strains of *S. cerevisiae* at concentrations of 1–2 μg/mL and exhibited similar CD patterns in both trifluoroethanol and tris(hydroxymethyl)aminomethane buffer, pH 7.4. In contrast, the L-Ala^9 and L-Leu^9 analogues were more than 200 times less active in the morphogenesis assay and had markedly different CD spectra. These results demonstrate that the position 9 residue plays an important role in determining the biological activity and solution conformation of α-factor. We suggest the presence of a type II β-turn in the Lys^7–Gln^{10} region when the α-factor assumes its biologically active conformation.

Sexual conjugation between haploid cells of *Saccharomyces cerevisiae* is induced by peptide pheromones, termed *a* factor and α-factor. The α-factor, Trp-His-Trp-Leu-Gln-Leu-Lys-Pro-Gly-Gln-Pro-Met-Tyr, is secreted by *MAT* α-cells and upon interaction with *MATa* cells inhibits DNA replication and cell division (Throm & Duntze, 1970). The dodecapeptide α-factor (lacking N-terminal Trp) is also biologically active. Detailed studies on structure–activity relationships in tridecapeptide and dodecapeptide pheromones have been reported

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