Characteristics of a Copper-Dependent Cross-Linking Reaction Between Two Forms of Cytochrome *P*-450 in Rabbit-Liver Microsomal Membranes

Peter R. McINTOSH and Robert B. FREEDMAN

Biological Laboratory, University of Kent, Canterbury, Kent CT2 7NJ, U.K.

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1. In liver microsomal membranes from adult rabbits treated with β -naphthoflavone, reaction with Cu^{2+} salts plus 1,10-phenanthroline leads to the cross-linking of the two specifically β -naphthoflavone-inducible cytochrome *P*-450 species, form 4 and form 6, to form homo- and hetero-dimer species. 2. The cross-linking is not reversed by treatment with 2-mercaptoethanol, so that it can be observed conveniently and specifically on conventional reducing sodium dodecyl sulphate/polyacrylamide gels. 3. The reaction occurs rapidly, and significant cross-linking is observed after 30 s at all temperatures from -10 to 40°C . 4. The cross-linking can be brought about by Cu^{2+} alone at concentrations greater than 0.5 mM , but not by 1,10-phenanthroline alone; at low Cu^{2+} concentrations, 1,10-phenanthroline enhances the cross-linking reaction, but high concentrations of 1,10-phenanthroline are inhibitory; the optimal molar ratio of Cu^{2+} to 1,10-phenanthroline is 4:1.5. The effect of Cu^{2+} is not mimicked by Mn^{2+} , Fe^{3+} , Fe^{2+} , Co^{2+} , Ni^{2+} , Zn^{2+} or Ag^+ ; Cu^+ is probably also ineffective. 6. The cross-linking reaction is inhibited by the prior addition of high concentrations of EDTA or thiol compounds, by sodium dodecyl sulphate at $\geq 0.1\%$ and by sodium deoxycholate and non-ionic detergents at $\geq 1\%$; the reaction cannot be reversed by incubation with EDTA or with thiol compounds after reaction with cupric phenanthroline; the cross-linking reaction is not inhibited by prior treatment of microsomal membranes with *N*-ethylmaleimide. 7. The chemical nature of the cross-linking reaction is unknown, but it is most unlikely that it involves the formation of intermolecular disulphide bonds. 8. The great specificity of the reaction makes it a promising tool for the study of molecular interactions between cytochrome *P*-450 species in intact microsomal membranes.

The molecular organization of the enzymic components of hepatic endoplasmic reticulum remains the subject of much speculation (Depierre & Dallner, 1975; Depierre & Ernster, 1977). The membrane comprises more than 50 proteins (Kaderbhai & Freedman, 1978) and is responsible for numerous metabolic processes, many of them multi-enzyme processes. An important example is the drug- and carcinogen-metabolizing mono-oxygenase system, which comprises a number of different forms of cytochrome *P*-450 and the flavoprotein NADPH-cytochrome *P*-450 reductase. In particular, three aspects of the organization of component proteins of the mono-oxygenase system need to be resolved: (i) whether the various forms of cytochrome *P*-450 and the reductase associate with each other in a stable fashion, or simply encounter

each other transiently (e.g. Yang, 1975; Peterson *et al.*, 1978); (ii) whether the NADH-linked electron-transport chain involving cytochrome *b*₅ interacts with the mono-oxygenase system (Archakov & Devichensky, 1975; Jansson & Schenkman, 1977); (iii) whether ancillary drug-metabolizing enzymes of the endoplasmic reticulum, such as epoxide hydratase (EC 3.3.2.3) and UDP-glucuronosyltransferase (EC 2.4.1.17) are topographically associated with the mono-oxygenase system (Depierre & Ernster, 1977; Seidegård *et al.* 1978).

In theory, studies with chemical cross-linking agents can resolve these questions, although there are a number of difficulties in extending cross-linking techniques from simpler problems to the analysis of membrane organization (Peters & Richards, 1977; Freedman, 1979). These difficulties arise mainly from the large number of distinct proteins present in most membranes and from the possibility of rapid

Abbreviation used: SDS, sodium dodecyl sulphate.

lateral movement of proteins, which makes it difficult to distinguish products generated by the cross-linking of proteins associated in long-term stable complexes from cross-linked products arising from 'random' collisions.

These difficulties are exemplified by our preliminary attempts to use the conventional cross-linking reagent, dimethyl suberimidate, to probe the molecular organization of rat liver microsomal membranes. Comparison of the distribution of microsomal proteins separated by SDS/polyacrylamide-gel electrophoresis after treatment of microsomal membranes with dimethyl suberimidate at 20 or 37°C with that of untreated membranes, revealed a decrease in resolution, a loss of material from all protein bands, and an increased appearance of aggregated material unable to enter 7.5% (w/v) polyacrylamide gels. There was no evidence to suggest the formation of discrete new cross-linked oligomeric molecular species. This observation demonstrates the general reactivity of microsomal proteins towards this reagent, a bisimide reactive to many nucleophiles, particularly lysine side chains. The formation of high-molecular-weight aggregates may reflect free mobility of many protein components of the membrane or extensive stable protein-protein contacts within the membrane.

In an attempt to produce more specific cross-linking, we have studied the effects on liver microsomal membranes of mild oxidants, which should cross-link proteins with free thiol groups by the formation of intermolecular disulphide bonds. In the course of such studies we observed that one of the oxidizing agents used [cupric salts plus 1,10-phenanthroline (cupric phenanthroline)] generates several cross-linked species that are not reduced by 7% (v/v) 2-mercaptoethanol, and are therefore unlikely to result from an oxidative mechanism of cross-linking (McIntosh & Freedman, 1978). This unusual reaction occurs only in liver microsomal membranes isolated from adult rabbits pretreated with β -naphthoflavone, an established inducer of particular forms of cytochrome *P*-450 (Haugen *et al.*, 1975), and not to any significant extent in microsomal preparations from control or phenobarbital-pretreated animals. We have since shown that the cross-linked species are generated from two forms of cytochrome *P*-450 induced by β -naphthoflavone, namely form 4 and form 6 cytochrome *P*-450 (McIntosh & Freedman, 1979).

The remarkable specificity of the reaction means that the interactions of these forms of cytochrome *P*-450 with themselves and with each other can be studied *in situ* in intact microsomal preparations. We describe here the principal characteristics of this unusual cross-linking reaction; some results have been presented in preliminary form elsewhere (McIntosh & Freedman, 1978).

Materials and Methods

Animals

Male New Zealand White rabbits obtained from Hop Rabbits Ltd., Chilham, Kent, U.K. were used. Three groups of three animals were employed: group A were of median age 45 days and weight 1.1–1.5 kg; group B were of weight 2.2–2.4 kg; group C were of median age 98 days and weight 2.8–3.7 kg.

Treatment with β -naphthoflavone

Group A and C animals were injected intraperitoneally with a suspension of β -naphthoflavone (Aldrich Chemical Co., Gillingham, Dorset, U.K.) in Mazola corn oil to a final dosage of 60 mg/kg body wt., 24 h before being killed. Group B animals were similarly injected to a final dosage of 50 mg/kg body wt., 48 h before being killed.

Preparation of liver microsomal fractions

All animals were starved for 24 h before being killed by cervical dislocation. Microsomal fractions from the pooled livers of each group were prepared as follows by adaptation of the method of van der Hoeven & Coon (1974). (i) After removal of gall-bladders and rinsing in cold water, livers were homogenized with 4 ml of ice-cold TKE buffer (0.1 M-Tris, 0.1 M-KCl, 1 mM-EDTA; adjusted to pH 7.4 with acetic acid at 20°C)/g in an MSE AtoMix blender by 2 × 40 s bursts. (ii) Homogenates were centrifuged in an MSE 6 × 250 ml rotor for 30 min at 4°C and 10 000 g (r_{av} , 10.5 cm). (iii) Supernatant fractions were centrifuged in an MSE 10 × 100 ml rotor for 3 h at 4°C and 45 000 g (r_{av} , 9.5 cm). (iv) Pellets from the above operation were resuspended in a volume of pyrophosphate buffer (0.1 M-sodium pyrophosphate, 1 mM-EDTA; adjusted to pH 7.4 with acetic acid at 20°C) equal to the original volume of TKE buffer, in a loose-fitting Potter homogenizer. Suspensions were recentrifuged as in (iii). (v) Operation (iv) was repeated once. (vi) The final washed pellets of microsomal membranes were resuspended in a small volume of TKEG buffer [0.01 M-Tris, 0.1 M-KCl, 1 mM-EDTA, 20% (v/v) glycerol; adjusted to pH 7.4 at 20°C with acetic acid] to final protein concentrations of 15–25 mg/ml. (vii) The suspensions were divided into small portions and frozen at –20°C until required. We have confirmed that the cross-linking effects described in this communication occur in fresh as well as in frozen material. Samples may be stored for up to several months before use.

Protein concentrations were determined by the method of Lowry *et al.* (1951), with bovine serum albumin in TKEG buffer as a standard.

Incubations with cupric phenanthroline

Incubations were performed in 1 ml plastic Eppendorf tubes. Thawed microsomal suspensions in TKEG buffer were diluted with distilled water or 20% glycerol (when so stated) to final protein concentrations of 2–8 mg/ml. Aqueous solutions of CuCl_2 and 1,10-phenanthroline (BDH Chemicals, Poole, Dorset, U.K.; stock solution 2.5 mM) were added to initiate the cross-linking reaction. Precise conditions of reagent concentration, temperature and time varied between experiments and are reported individually in Figure legends. Incubation volumes varied from 100 to 300 μl .

Incubations were terminated by the addition of a large volume of SDS-containing buffer [0.25 M-Tris/8% (w/v) SDS; adjusted to pH 6.8 at 20°C with acetic acid] such that final solutions contained 5($\pm$ 0.5)% SDS. This termination procedure is effective at room temperature or on ice.

Sample preparation for electrophoresis

All terminated incubations were supplemented with the addition of glycerol to a final concentration 20% (v/v) and the dye Bromophenol Blue (Fison's, Loughborough, Leics., U.K.; aqueous stock solution 0.1%, w/v), to a final concentration of 0.003% (w/v).

In addition, unless otherwise stated, all samples were reduced by the addition of 2-mercaptoethanol to a final concentration of 7($\pm$ 0.8)% (v/v).

These conditions caused an increase in volume of the samples of approx. 30%. This increase resulted in a final SDS concentration of approx. 3.5% (w/v).

Electrophoresis

Electrophoresis of the SDS-solubilized microsomal membrane fractions was on polyacrylamide slab gels of 1.5-mm thickness, by modification of the method of Laemmli (1970).

The constitution of the 7.5% polyacrylamide separating gels used was 10 ml of stock acrylamide solution [30% (w/v) acrylamide (BDH; specially pure for electrophoresis), 0.8% (w/v) NNN' -methylenebisacrylamide (BDH; specially pure for electrophoresis)], 10 ml of separating gel buffer [1.5 M-Tris/0.4% (w/v) SDS; adjusted to pH 8.8 at 20°C with HCl] and 20 ml of water. Polymerization was achieved by the addition of 50 μl of NNN' -tetramethylethylenediamine (BDH) and 250 μl of a freshly prepared 10% (w/v) solution of ammonium persulphate.

The separating gel was overlaid with 10–15 mm of 3% polyacrylamide stacking gel of the following constitution, into which combs of 12 or 18 slots were introduced before polymerization: 1 ml of stock acrylamide solution, 2.5 ml of stacking gel buffer (0.5 M-Tris/0.4% SDS; adjusted to pH 6.8 at 20°C with HCl) and 6.5 ml of water. Polymeriza-

tion was with 15 μl of NNN' -tetramethylethylenediamine and 75 μl of 10% ammonium persulphate.

Buffer used in both electrode compartments contained 0.05 M-Tris base, 0.384 M-glycine and 0.2% (w/v) SDS. This buffer is not adjusted to a particular pH.

Samples (10–30 μl) containing approx., 20 μg of microsomal membrane protein were loaded into each slot. Gels were electrophoresed for 3–3½ h at 30 mA/gel on a constant-current setting. Maximum permitted voltage was set at 150 V. After several hours, voltage became limiting. Migration of the Bromophenol Blue marker was approx. 10 cm.

Gels were stained overnight for protein in propan-2-ol/acetic acid/water (5:2:13, by vol.) containing 0.05% (w/v) Coomassie Brilliant Blue R (Gurr/Hopkin and Williams, Chadwell Heath, Essex, U.K.). The gel background was then cleared by repeated changes of 10% (v/v) acetic acid.

Densitometric scanning of gels and normalization of scan profiles

Tracks were excised from the gels and scanned at 570 nm in a Gilford 240 spectrophotometer with a gel-scanning device.

The amounts of protein loaded into each slot were not always identical owing to inaccuracies in pipetting, particularly of viscous glycerol solutions. Before comparison of densitometric scans can be properly made, it is necessary to perform a normalization procedure. For this purpose we have selected two bands, N1 and N2 (see Figs. 2 and 3), that are not altered during incubation with the cross-linking agents used in this study. Estimates of areas of the densitometric curves (i.e. a, b, c in Fig. 3a) associated with bands A, B and C have been expressed after normalization with respect to the appropriate area (n1) associated with band N1 in the same track. Areas d and e (Fig. 3b) have been normalized to area n2.

Results and Discussion

Generation of cross-linked dimers of cytochrome P-450 species by treatment of microsomal membranes with cupric phenanthroline

Reaction of liver microsomal membranes from β -naphthoflavone-induced rabbits with cupric phenanthroline leads to significant changes in the band pattern when the membrane proteins are analysed in non-reducing SDS/polyacrylamide gels in which thiol-disulphide interchange is prevented (Fig. 1, tracks a and b). It is natural to conclude that these changes produced by cupric phenanthroline, an oxidizing agent, are due to the formation of intermolecular disulphide bonds, in which case the changes should be reversed by thiols, and would not be noted on conventional reducing SDS/polyacryl-

Fig. 1. Diagrammatic representation of the effect of cupric phenanthroline on microsomal membrane proteins analysed by SDS/polyacrylamide gel electrophoresis in non-reducing (tracks *a* and *b*) and reducing (tracks *c* and *d*) conditions

Microsomal membranes (8 mg/ml) from group A rabbits were incubated at 4°C for 20 min in the presence (*b* and *d*) or absence (*a* and *c*) of 0.33 mM-CuCl₂ + 0.33 mM-1,10-phenanthroline (CuP). Samples were subsequently incubated for 15 min in the presence (*a* and *b*) or absence (*c* and *d*) of 10 mM-*N*-ethylmaleimide. SDS-containing buffer was added to stop the reaction. 2-Mercaptoethanol was added to samples *c* and *d* to a final concentration of 7% (v/v) before electrophoresis.

amide gels (Steck, 1972; Peters & Richards, 1977). However, when the same cupric phenanthroline-treated microsomal preparations are analysed on SDS/polyacrylamide gels containing 2-mercaptoethanol, three distinct new bands (A, B and C) are noted compared with patterns obtained from untreated microsomal fractions run in the same conditions (Fig. 1, tracks *c* and *d*). The other changes produced by cupric phenanthroline are reversed under these conditions, so that the reducing gels display a very specific effect of cupric phenanthroline, which appears to be the formation of cross-linked species not involving intermolecular disulphide bond formation. The present paper deals exclusively with this specific effect observed on reducing gels.

The only changes evident in the pattern of microsomal proteins separated by SDS/polyacrylamide gel electrophoresis under reducing conditions after the incubation of microsomal membranes

with cupric phenanthroline are the appearance of new bands A, B and C and loss in intensity of two bands D and E (Fig. 1, tracks *c* and *d*; Fig. 2). The material contributing to the 'new' bands must therefore be derived from material lost from bands D and E, or from material that fails to enter the gel. This latter possibility, however, can be ruled out, since the amount of such material in gels of untreated microsomal preparations is insignificant, and certainly insufficient to account for the large increases in bands A, B and C.

We have previously shown that no comparable effects are observed on cupric phenanthroline treatment of microsomal fractions from control or phenobarbital-induced rabbits; specific β -naphthoflavone-induced proteins must be present at positions D and E in order for the cross-linking reaction to occur (McIntosh & Freedman, 1978). We have also shown that these species are two forms of cytochrome *P*-450 which, in the nomenclature of Norman *et al.* (1978), are form 6 cytochrome *P*-450 (D) of estimated mol.wt. 57 000 and form 4 cytochrome *P*-450 (E) of estimated mol.wt. 53 000 (McIntosh & Freedman, 1979). The estimated molecular weights of the new bands, A, B and C, are approximately twice those of the cytochrome *P*-450 species, being 107 000, 103 000 and 100 000 respectively, strongly suggesting that they represent cross-linked dimers of these proteins. On the basis of the variation with age of the inducibility of forms 6 and 4, we have established that A is the homodimer of form 6 cytochrome *P*-450 (i.e. A = form 6-form 6), and inferred that C represents the homodimer of form 4 cytochrome *P*-450 (i.e. C = form 4-form 4), while B represents the heterodimer comprising both species (i.e. B = form 4-form 6) (McIntosh & Freedman, 1979).

Kinetics of the cross-linking reaction

The cross-linking reaction occurs rapidly, significant amounts of bands A, B and C being observed within 30 s of incubation (Fig. 2). Densitometric scans of portions of two tracks are shown in Fig. 3*a* to illustrate the increase in material at positions A, B and C after 15 min of incubation. Fig. 3*b* illustrates loss in material from positions D and E. In order to define the kinetics of the reaction in more detail, it is important to quantify the extent of reaction by estimation of the intensity of stained material. Because of variations in the total amounts of material loaded on to each track, it is necessary to carry out a normalization of each densitometric scan. This is done by reference to the stain intensity of peaks N1 and N2, which are not altered by treatment with cupric phenanthroline (see the Materials and Methods section).

The appearance of additional material at positions A, B, C and loss of material at positions D and

Fig. 2. An SDS/polyacrylamide gel demonstrating the time course of cross-linking with cupric phenanthroline. Microsomal membranes from group A rabbits (approx. 6 mg/ml in the presence of 20%, v/v, glycerol) were preincubated for 15 min at 26°C. CuCl_2 (0.4 mM) + 1,10-phenanthroline (0.1 mM) were then added and incubation was continued at the same temperature for the following times before addition of the terminating buffer: for tracks 1, 9, and 17, $t=0$ min; for tracks 2–8 and 10–16, $t=0.5, 0.75, 1, 1.5, 2, 3, 4, 5, 7.5, 10, 15, 30, 45, 60$ min respectively. 2-Mercaptoethanol (7%, v/v) was added to all samples before electrophoresis.

E as functions of incubation time, quantified by this procedure, are shown in Fig. 4. Increases in material at positions A and B reach maximal values within 30 s of incubation, and thereafter, intensities of bands A and B remain constant. Considerable amounts of material at position C are formed within 30 s also, but the intensity of this peak continues to rise slowly over a period of 5 min before reaching a stable maximum value. This could arise from a lower intrinsic rate for the reaction between form 4 cytochrome *P*-450 molecules, but it may be the case that in these conditions a slow diffusion or exchange process in the membrane is required to bring some form 4 molecules into contact. For example, the model of Peterson *et al.* (1978) envisages some cytochrome *P*-450 molecules in close contact, clustered round a molecule of cytochrome *P*-450 reductase, while others diffuse independently. Loss in material at positions D and E also occurs very rapidly. The intensity of band E continues to decrease slowly, even after no further increases can be detected in A, B, C. This trend continues if longer incubations are studied (results not shown) and probably reflects an instability of the form 4 cytochrome *P*-450 to incubation in these conditions, and the resultant generation of some form of aggregated material. Increased amounts of aggregated material unable to enter the 7.5% gel are seen after prolonged incubations (see Fig. 2). Specific higher oligomers such as trimers or tetramers of

cytochrome *P*-450 species have not yet been observed.

Quantitative estimates of material lost on cross-linking from positions D and E (i.e. $d + e$) correlate with estimates of the amount gained at positions A, B and C (i.e. $a + b + c$) to within $\pm 30\%$. The lack of precise agreement is almost certainly a reflection of inadequacies in the normalization procedure used. The adoption of more sophisticated procedures (e.g. those of McIntosh *et al.*, 1980) is unlikely to be justified in view of the intrinsic imprecision of densitometric scan data; sources of imprecision are distortions of band shape, failure to section gels precisely parallel with the direction of migration and slight vagaries in the mobilities of the same bands on different tracks. Bearing this reservation in mind, however, the methods of quantitative analysis employed here are adequate to monitor the large changes in band intensity that occur as a consequence of treatment with cupric phenanthroline. Measurement of peak heights on densitometric scans offers a simpler method of quantitative analysis than measurement of peak areas; the same trends in the behaviour of A, B and C with increasing incubation periods are seen using this method (Fig. 4b, cf 4a). The kinetics of the cross-linking reaction, and the maximum extent of cross-linking (maximum peak heights of bands A, B and C) are broadly similar at all temperatures between 0 and 40°C. At -10°C , which is in the region of the bulk lipid

Fig. 3. Densitometric scans demonstrating (a) the gain of stained material at positions A, B and C and (b) the loss at positions D and E

Portions of tracks from the gel shown in Fig. 2 were scanned (see the Materials and Methods section) for quantitation of the formation of peaks, A, B and C, and loss of peaks D and E after 15 min of reaction. N1 and N2 are reference peaks (see Materials and Methods); a, b, c, d, e, n1 and n2 are areas under peaks A, B, C, D, E, N1 and N2.

transition temperature of microsomal membranes (Blazyk & Steim, 1972), substantial cross-linking also occurs, although the maximum values of all

Fig. 4. Time course of the changes in intensity of protein bands A-E

(a) Quantitation from peak areas; (b) quantitation from peak heights. Data were derived from densitometric scans of tracks from the gel shown in Fig. 2; a-e are areas associated with bands A-E. See Fig. 3 for further details.

cross-linked peaks are reduced. We hope to present these findings more fully elsewhere as they are an integral part of current studies on the role of the membrane in interactions between components of the microsomal mono-oxygenase system.

Effects of different Cu^{2+} ion and 1,10-phenanthroline concentrations on the formation of the cross-linked species

We have previously shown that at a concen-

tration of 0.22 mM, 1,10-phenanthroline alone has no cross-linking effect, and that Cu^{2+} alone at the same concentration has only a slight effect (McIntosh & Freedman, 1978). Extensive formation of A, B and C was seen in the presence of both agents together at this concentration. Fig. 5 demonstrates that Cu^{2+} at concentrations ≥ 0.5 mM is quite effective at bringing about cross-linking in the absence of 1,10-phenanthroline; bands A, B and C are not seen in untreated controls or in samples treated at a lower concentration (0.1 mM) of Cu^{2+} . Some enhancement

of the Cu^{2+} -dependent cross-linking is seen if 1 mM-1,10-phenanthroline is included in the incubations; at this concentration of 1,10-phenanthroline, its enhancing effect is seen most strikingly in the presence of 0.5 mM- Cu^{2+} .

The effect of varying the concentration of 1,10-phenanthroline was studied in order to clarify its role in potentiating the Cu^{2+} -dependent cross-linking reaction (Fig. 6). In the presence of 0.44 mM- Cu^{2+} , increasing concentrations of 1,10-phenanthroline up to 0.1–0.2 mM give increasingly effective cross-linking, but higher concentrations produce a steadily decreasing enhancement of cross-linking. This behaviour is observed in a titration of 1,10-phenanthroline in the presence of a lower Cu^{2+} concentration (0.22 mM), but in this case the maximum enhancement is produced by a lower concentration of 1,10-phenanthroline (0.05–0.1 mM).

It is interesting that the maximum enhancement is observed in both cases at an approximate Cu^{2+} /phenanthroline molar ratio of 4:1. The standard complex ion formed between Cu^{2+} and this ligand is the square planar complex with a Cu^{2+} /ligand stoichiometry of 1:2 (Cotton & Wilkinson, 1972). It is clear that at the Cu^{2+} /ligand ratio that produces maximum enhancement of the cross-linking effect, only a small proportion of the Cu^{2+} ions will be in the complexed form. This fact, together with the observation that the cross-linking reaction can be brought about by moderate concentrations of Cu^{2+} alone, suggests that the conventional Cu^{2+} -bis-

Fig. 5. Diagrammatic representation of portions of gel tracks showing the effects of incubating microsomal membranes with varying concentrations of CuCl_2 . Microsomal membranes from group C rabbits (approx. 4 mg of protein/ml) were incubated for 15 min at 20°C with the concentrations of CuCl_2 shown, in the absence or presence of 1,10-phenanthroline (1 mM). 2-Mercaptoethanol was added to all samples after termination of the incubation.

Fig. 6. Graph showing the effect of incubating microsomal membranes with varying concentrations of 1,10-phenanthroline

Incubations were carried out as in the legend to Fig. 5, but in the presence of varying concentrations of 1,10-phenanthroline and either 0.22 mM- CuCl_2 (dashed lines) or 0.44 mM- CuCl_2 (full lines). All samples were treated with 2-mercaptoethanol prior to electrophoresis. Peak heights of A (Δ), B ($\square$) and C ($\circ$) were determined by densitometry.

(1,10-phenanthroline) complex is not the reactive species. It is possible that the presence of small quantities of the ligand facilitates the transfer of Cu^{2+} ions to a reaction site in the membrane; the chelate may pass more readily into non-polar regions of the membrane than the simple Cu^{2+} (aq.) cation.

There is some evidence that the relative extents of formation of the species A, B and C varies with the concentrations of Cu^{2+} and 1,10-phenanthroline. In the presence of 0.5 mM- Cu^{2+} , 1 mM-1,10-phenanthroline enhances the formation of bands A and B, but in the presence of 1 mM- or 5 mM- Cu^{2+} , the same concentration of 1,10-phenanthroline enhances the formation of band C only. Conversely, at a fixed concentration of Cu^{2+} (0.44 mM), low concentrations of 1,10-phenanthroline (<0.1 mM) stimulate the formation of bands A and B, whereas concentrations of 1,10-phenanthroline of about 0.1–0.2 mM markedly enhance the formation of bands B and C. We have not investigated this phenomenon more closely.

Metal-ion specificity of the cross-linking reaction

The following salts were tested for cross-linking activity: MnCl_2 , FeCl_3 , FeSO_4 , CoCl_2 , NiCl_2 , CuCl ,

CuCl_2 , CuSO_4 , ZnSO_4 and AgCl (Fig. 7). CuSO_4 proved as effective as CuCl_2 in bringing about the appearance of bands A, B and C, as expected. All other salts were ineffective, with the possible exception of CuCl . The sample of CuCl used was almost certainly contaminated with Cu^{2+} ions, since it was pale green in colour, whereas pure CuCl is white. It thus appears very likely that the cross-linking reaction is specific to the Cu^{2+} ion.

Effect of EDTA on cross-linking

The appearance of the cross-linked dimers A, B and C is completely suppressed by the addition of EDTA to a high concentration (27 mM) before incubation of microsomal fractions with cupric phenanthroline (results not shown). This suggests that chelation with EDTA prevents the Cu^{2+} from bringing about cross-linking. The addition of the same amount of EDTA after incubation of microsomes with cupric phenanthroline did not reverse cross-linking, indicating that if Cu^{2+} ions are directly involved in intermolecular bonding as components of the cross-linked species, they are not at all readily dissociable.

Fig. 7. Gel demonstrating the effect of incubating various metal salts with microsomal membranes in the presence of 1,10-phenanthroline

All salts except CuCl and AgCl were added to a final concentration of 0.66 mM. A few grains of the insoluble salts CuCl and AgCl were used. Incubations of microsomal membranes (approx. 4 mg of protein/ml) from group C rabbits were at 22°C for 20 min and included 0.16 mM-1,10-phenanthroline. 2-Mercaptoethanol was added to all samples prior to electrophoresis.

Effects of detergents on extent of cross-linking

The dependence of the cross-linking reaction on membrane integrity was studied using detergents. An early finding was that the cross-linking reaction with cupric phenanthroline does not occur in the presence of a high concentration (5%) of the anionic detergent sodium dodecyl sulphate (McIntosh & Freedman, 1978); this effect is now used to stop cross-linking at the end of test incubations. Fig. 8 shows the effects on cross-linking of the presence of various concentrations of several detergents. The inhibitory effect of SDS is first seen at 0.1% concentration and is complete at 1% and higher concentrations. Sodium deoxycholate and the non-ionic detergents Renex 690 and Triton N-101 were less effective at inhibiting the reaction; in all cases 1% concentrations only partially inhibited cross-linking. Sodium deoxycholate was slightly effective at 0.1%, but the non-ionic detergents were entirely ineffective at this concentration.

Evidence suggesting that protein thiol groups are not involved in the cross-linking reaction

N-Ethylmaleimide is widely used as a specific reagent for the modification of protein thiol groups (Riordan & Vallee, 1977). A number of experiments have shown that the characteristic cross-linking brought about by cupric phenanthroline in microsomal fractions is not inhibited by their treatment with excess *N*-ethylmaleimide (10 mM) either before or after the incubation with cupric phenanthroline (results not shown). This implies that protein thiol groups are not involved in the cross-linking reaction unless such thiol groups are inaccessible to *N*-ethylmaleimide though reactive to cupric phenanthroline.

Conclusions

The following conclusions can be drawn from the

characteristics of this unusual cross-linking phenomenon.

(i) *The reaction generates homo- and heterodimers of the cytochrome P-450 species form 4 and form 6.* Findings leading to this conclusion have been summarized elsewhere (McIntosh & Freedman, 1979), and the data presented here entirely support this conclusion.

(ii) *The reaction does not involve intermolecular disulphide bond formation.* The evidence for this claim is as follows: (a) the cross-linked dimers are regularly observed after electrophoresis of samples prepared in >6% (v/v) mercaptoethanol and >3% (w/v) SDS; (b) the cross-linking is not reversed even under more extreme reducing conditions, namely, boiling for 2 min in the presence of 0.28 M-dithiothreitol; (c) pretreatment of microsomal fractions with the thiol-blocking reagent *N*-ethylmaleimide does not prevent formation of the cross-linked species A, B and C; (d) in preliminary experiments we have observed that the mild oxidant sodium tetrathionate, at 20 mM and a wide variety of temperatures, does not promote the formation of these species, although this reagent is widely used to cross-link proteins through the formation of intermolecular disulphide bonds (Haest *et al.*, 1977).

(iii) *The cross-linking reaction is rapid.* For two of the dimeric species, cross-linking is complete within 30 s at 26°C, and the majority of the third cross-linked species is also formed within the first 30 s. But the time-scale in these experiments is too slow to enable clear conclusions to be drawn as to whether the reacting species participate in long-term associations in the membrane or whether reaction occurs when freely diffusing cytochrome molecules collide.

A model in which cross-linking occurs on collision is certainly consistent with our data, and it is attractive to explain the slow phase in the formation of dimer C, in particular, as being determined by a slow diffusion or exchange process. However, it is

Fig. 8. Diagrammatic representation of the effects on the formation of bands A, B and C of the presence of various detergents during incubation of microsomal membranes with cupric phenanthroline

Microsomal membranes (approx. 2 mg of protein/ml) from group C rabbits were incubated with 2 mM- CuCl_2 + 0.75 mM-1,10-phenanthroline at 21°C for 15 min. Sodium dodecyl sulphate, Renex 690, Triton N-101 and sodium deoxycholate were added to the indicated concentrations (% w/v), before the initiation of reaction. Detergent was omitted from control tracks x and y; these samples were incubated either in the absence (track x) or presence (track y) of cupric phenanthroline.

unlikely that all the observed cross-linking results from random collisions between freely diffusing species. Significant extents of cross-linking have been observed at temperatures down to -15°C (P. R. McIntosh & R. B. Freedman, unpublished observations); for rat liver microsomes this is below the lipid phase transition temperature (Blazyk & Steim, 1972), a temperature at which lateral movements of proteins would be expected to cease. The fact that cross-linking is restricted to the cytochromes induced by β -naphthoflavone obviously suggests that these two species have some structural features in common, but the observation that cross-linking is so extensive and rapid over a wide range of temperature lends weight to the suggestion that these inducible cytochromes may share a specific micro-environment in the membrane, as envisaged in the model of Peterson *et al.* (1978).

(iv) *The reaction is inhibited by high concentrations of detergents.* In decreasing order of effectiveness cross-linking is inhibited by sodium dodecyl sulphate > sodium deoxycholate > Triton N-101 = Renex 690. This inhibition probably reflects a requirement for membrane integrity for cross-linking to occur. However, it is not easy to rule out the possibility that the detergents have direct effects on the conformations of the individual proteins which alter the reactivity or accessibility of groups involved in the cross-linking reaction.

(v) *The mechanism of cross-linking remains unknown but the reaction is specific to the Cu^{2+} ion.* The reaction is totally dependent on the presence of Cu^{2+} ions and is inhibited by high concentrations of EDTA and 1,10-phenanthroline; closely related cations will not replace Cu^{2+} . While the formation of intermolecular disulphide bonds can be eliminated, there is not yet sufficient evidence to describe the nature of the cross-link formed between the cytochrome *P*-450 species. Related, but not identical, observations have recently been made with the $(\text{Na}^{+} + \text{K}^{+})$ -ATPase of dog kidney plasma membrane (Huang & Askari, 1978). In this case a Cu^{2+} -dependent cross-linking has been observed, but this is reversed by EDTA and blocked by pre-treatment with *N*-ethylmaleimide, in contrast to the observations made in our system.

Only dimeric species have been observed to be generated by cross-linking, even when samples are analysed on SDS/polyacrylamide gels of low acrylamide concentrations on which all oligomers of the interacting cytochrome *P*-450 species up to hexamers would be detected (P. R. McIntosh & R. B. Freedman, unpublished observations). So it seems probable that only one interaction site for cross-linking exists on each molecule of the two forms of cytochrome *P*-450.

Since we have not observed similar non-oxidative cross-linking upon cupric phenanthroline treatment

of microsomes from rats (either untreated or induced with the polycyclic aromatic hydrocarbon, 3-methylcholanthrene), it may be that, among the numerous rat and rabbit microsomal proteins, only these particular rabbit cytochrome *P*-450 species possess the necessary molecular features for cross-linking to occur. The restriction of the reaction to only two proteins in microsomes means that the interaction of these cytochrome *P*-450 molecules can be studied in intact microsomes, in which the integrity of the membrane is undisturbed, without the necessity for purification of the interacting species. Further studies of this cross-linking phenomenon may therefore be of great value in probing the molecular structure of the microsomal mono-oxygenase system.

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