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UNUSUAL REDOX BEHAVIOUR OF CYTOCHROME *b*-561 FROM BOVINE CHROMAFFIN GRANULE MEMBRANES

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(1) Redox titrations of cytochrome *b*-561 have been performed with the purified cytochrome and with intact and detergent-solubilized chromaffin-granule membranes. (2) The midpoint redox potential of the cytochrome is 100–130 mV; this depends upon the composition of the buffer, but is independent of pH in the range 5.5–7.5; partial proteolysis of the cytochrome raises the midpoint potential to 160 mV. (3) The Nernst plots of titration data have slopes of 75–115 mV, and are in some cases sigmoid in shape. This may be explained by negative cooperativity during redox transitions in oligomeric cytochrome *b*-561. (4) Measurements of the haem and cytochrome content of chromaffin granule membrane suggest a haem content of 1 mol / mol protein. (5) Chemical crosslinking of cytochrome *b*-561 suggests that it may exist as an oligomer of 4–6 polypeptide chains within the chromaffin granule membrane. Aggregation of purified cytochrome *b*-561 was shown by gel filtration studies and by immunological methods in SDS-polyacrylamide gels. Studies of the molecular weight of the aggregates suggest that the monomer has a molecular weight close to 22000, but migrates anomalously slowly during electrophoresis.

Introduction

The catecholamine-storage granules of the adrenal medulla (known as chromaffin granules), the noradrenaline-containing vesicles of splenic nerve and the 5-hydroxytryptamine-containing dense granules of platelets contain a membrane-bound *b*-type cytochrome, termed *b*-561 because of the characteristic maximum in its reduced/oxidized difference spectrum [1–3]. In chromaffin granules, the concentration of this cytochrome has been reported to be as high as 7.4 nmol/mg membrane protein, equivalent to 15–20% of the total membrane protein [4]. Cytochrome *b*-561 is now known to be identical with the major membrane component previously known as chromomembrin B [5], and has been shown by chemical labelling, proteolytic digestion and immunological techniques to be a transmembrane protein [6,7]. The cytochrome has been purified from bovine chromaffin granule membranes after solubilization with various detergents [5,8,9]: reported values for its molecular weight are between 20500 and 30000 [7,9,10], and its isoelectric point is 6.2.

Despite recent progress in the structural characterization of cytochrome *b*-561, its function is still unknown. In catecholamine-storing granules, catecholamine biosynthesis requires transmem-

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Abbreviations: $C_{12}E_8$, octaethyleneglycoldodecylether; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; Mes, 4-morpholineethanesulphonic acid; Mops, 4-morpholinepropanesulphonic acid; Bicine, *N*,*N*-bis(2-hydroxyethyl)glycine.

brane electron transport, as formation of noradrenaline is catalysed by the intragranular mixed-function oxygenase dopamine β-hydroxylase (EC 1.14.17.1). Inward transport of electrons (from external ferrocyanide to internal dopamine β-hydroxylase) and also outward transport (from internal ascorbate to external ferrocyanide) have been demonstrated in resealed chromaffin-granule 'ghosts' [11,12], but in neither case has cytochrome *b*-561 been directly implicated. Discussion of the role of cytochrome *b*-561 has been based upon its reported midpoint redox potential of +140 mV [13]. Since chromaffin granules maintain a transmembrane pH gradient of approx. 1.5, acid inside [14], we set out to investigate in more detail the redox properties of cytochrome *b*-561, and in particular the effect of pH on its midpoint redox potential.

Materials and Methods

Chromaffin granules and chromaffin granule membranes were prepared from bovine adrenal glands as described previously [15]. Cytochrome *b*-561 was purified by a published procedure [5], using a preparation of aminohexyl-Sepharose with a low degree of substitution (approx. 0.5 μmol/ml gel). Antisera to this cytochrome were raised in rabbits, by conventional means . Cytochrome *c*-555 from *Chlorobium thiosulphateophilum* was the gift of Dr. T.E. Meyer, University of California. Membranes were prepared for redox titration by dialysis against three changes of 100 vol. of buffer, over 48 h at 4°C. In some cases the membranes were solubilized before dialysis by addition of the nonionic detergent $C_{12}E_8$ (Kouyoh Chemical, Tokyo) to a final concentration of 0.5%.

Spectrophotometric redox titrations were performed essentially as described by Dutton [16], in a Unicam SP1800 recording spectrophotometer. The cuvette was equipped with a combined platinum and Ag|AgCl electrode (Russell pH, Auchtermuchty, U.K.) and was continuously stirred while the surface of the solution was flushed with argon. Samples of cytochromes *b*-561 and *c*-555 contained the following mediators (each 20 μM): 2,3,5,6-diamino-*p*-phenylenediamine, duroquinol, 2-hydroxy-1,4-naphthoquinone, phenazine ethosulphate and phenazine methosulphate. In addition, 20 μM Fe^{2+} and 400 μM EDTA were added. The buffer concentration was 0.05 M, the temperature was approx. 20°C, and in some experiments $C_{12}E_8$ (0.5%) was included. Before titration, samples were fully oxidized with ferricyanide, then flushed with argon for 1 h. Reductive titration was by addition of small (0.1–1.0 μl) aliquots of sodium dithionite (50 mM); for subsequent oxidative titration, the titrant was potassium ferricyanide (50 mM). Before recording the spectrum, the absorbance was adjusted to a constant value at the isosbestic point of the cytochrome: 569 nm for cytochrome *b*-561, 544 nm for *c*-555 and 540 nm for *c*-550. Horse-heart cytochrome *c*-550 (Sigma) was titrated aerobically, without the addition of mediators.

Exclusion chromatography was performed on a column of Bio-Gel P-100 (53 × 2.5 cm) at room temperature (approx. 18°C) with a flow rate of 0.12 ml/min. Eluted fractions of 1.15 ml were collected, and the elution volume of proteins determined by gel electrophoresis of each sample.

Crosslinking of membrane proteins was performed with purified, dialysed chromaffin granule membranes. All crosslinking reagents were supplied by Pierce. The conditions of crosslinking were: 2 mg/ml protein/0.05 M bicine-NaOH buffer (pH 8.6). Membranes were incubated with various concentrations of dimethyl suberimidate or disuccinimidyl suberate at 30°C, or preincubated with various concentrations of 4-azidobromacetophenone or 4-fluoro-3-nitrophenyl azide for 1 h at 30°C in the dark, before ultraviolet irradiation at 0°C. After crosslinking, protein samples were precipitated with 10 vol. acetone/ethanol (1 : 1) at 0°C, collected by centrifugation and dissolved in electrophoresis sample buffer.

For proteolytic digestion of cytochrome *b*-561, crude chromaffin granules were incubated overnight at 18°C with 0.1 mg/ml chymotrypsin/0.3 M sucrose/10 mM Hepes-NaOH (pH 7.0). Membranes were prepared from protease-treated granules by the usual procedure, all buffers containing 0.1 mM phenylmethylsulphonyl fluoride.

Polyacrylamide gel electrophoresis in one or two dimensions was performed as described previously [5]. Electrophoretic transfer of proteins to cellulose nitrate and decoration with antiserum was as described by Towbin et al. [17]. Immune

complexes were detected by further decoration with 1 μg/ml *Staphylococcus aureus* protein A (Pharmacia), iodinated by the method of Tejedor and Ballesta [18] to a specific radioactivity of 20 MBq/mg. Autoradiography was on Agfa-Gevaert Curix RP-1 film.

Protein was assayed as described previously [5]. Cytochrome *b*-561 was assayed by difference spectroscopy, using an extinction coefficient of $23.3 \cdot 10^3$ cm^2/mol [8]. Haem was determined by the pyridine haemochrome method [19,20].

Results

Redox potentiometric data

The Nernst plots obtained by redox titration of purified cytochrome and of chromaffin granule membranes are shown in Fig. 1A: there is a good agreement between points obtained during reductive and oxidative titration, but the plots show several unusual features, which are discussed below. For comparison, the redox titration curve of the soluble monohaem cytochrome *c*-555 from *C. thiosulphatophilum* is presented in Fig. 1B.

Some experimental difficulties were encountered in performing redox titration of membrane-bound cytochrome *b*-561: these were light-scattering by the membrane suspension, and redox buffering, probably by catecholamines. Even after extensive dialysis the membranes contained significant concentrations of catecholamines, which could not be further depleted unless the membranes were solubilized with detergent [11]. In both reductive and oxidative titrations, the potential change brought about by addition of the titrant tended to reverse slowly, presumably through slow reduction or oxidation of membrane-bound catecholamines. Spectra were recorded only after the potential had become stable. The results of titrations performed in the presence and absence of detergent are shown in Table I, which also includes results obtained at several different pH values, and with membranes purified from chromaffin granules which had been treated with chymotrypsin.

The reduced/oxidized difference spectra obtained during reductive titration of purified cytochrome *b*-561 are shown in Fig. 2. The peak shows considerable asymmetry about its maximum at 561 nm, which might be taken as evidence for the presence of more than one cytochrome. These spectra were analysed by measuring the peak width

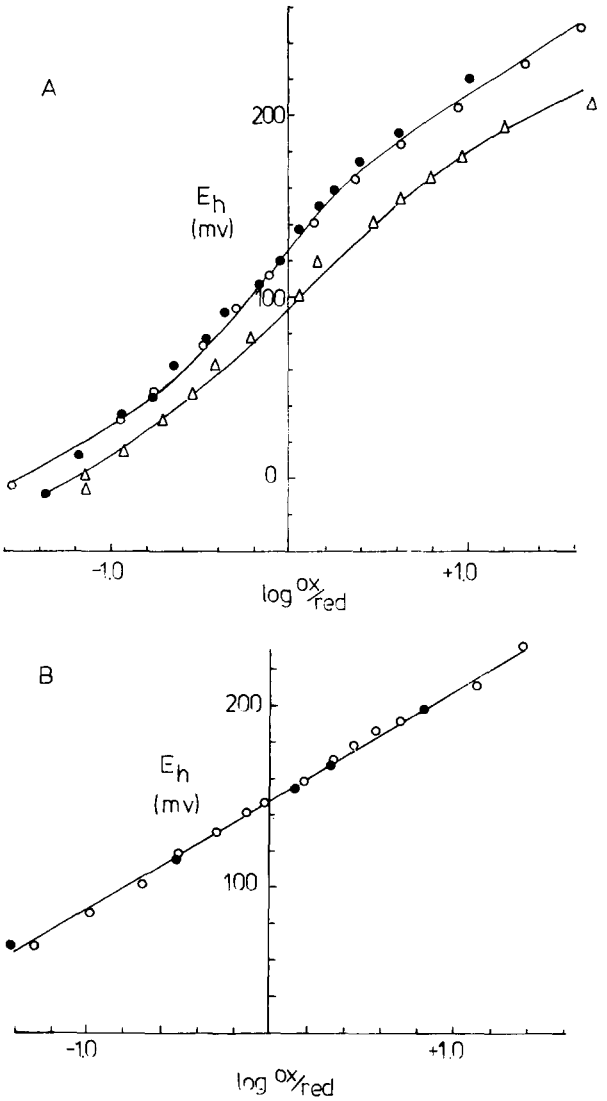

Fig. 1. (A) Upper curve: Nernst plot for oxidative (●) and reductive (○) titration of purified cytochrome *b*-561. The solid line is a theoretical curve for two components with E_m 170 and 70 mV, in the relative proportions 55:45. Lower curve (△): Nernst plot for reductive titration of membrane-bound cytochrome *b*-561. The solid line is a theoretical curve for two components with E_m 135 and 55 mV, in equal proportion. In both titrations the buffer was 0.05 M Mops (pH 7.1); other details are given in the text. (B) Nernst plot for oxidative (●) and reductive (○) titration of cytochrome *c*-555 from *C. thiosulphatophilum*. The solid line has slope 59 mV and E_m 148 mV. The buffer was 0.05 M Mops (pH 7.0).

TABLE I

VALUES OF THE MIDPOINT REDOX POTENTIAL AND SLOPE OF THE NERNST PLOT, OBTAINED BY TITRATION OF CYTOCHROME *b*-561 UNDER VARIOUS CONDITIONS

Cytochrome preparation	Detergent	Buffer	pH	Apparent midpoint potential (mV)	Slope (mV)
Purified	$C_{12}E_8$	Mops	7.1	128	sigmoid
	$C_{12}E_8$	Hepes	7.1	172 [a]	76 [a]
Membranes	–	Mes	5.7	133 [a]	87 [a]
	–	Mes	6.4	135	80
	–	Mops	6.2	115	84
	–	Mops	7.1	99	88
	–	Hepes	7.1	101 [a]	86 [a]
	–	Hepes	7.7	90	74
Membranes	$C_{12}E_8$	Mops	6.1	140	85 [b]
	$C_{12}E_8$	Mops	6.8	130	97 [b]
	$C_{12}E_8$	Mops	6.9	140	115
	$C_{12}E_8$	Hepes	7.1	137	89
	$C_{12}E_8$	mixed [c]	5.1	152	90
	$C_{12}E_8$	mixed [c]	5.8	170	108
	$C_{12}E_8$	mixed [c]	6.1	163	100
	$C_{12}E_8$	mixed [c]	6.9	146	100
	$C_{12}E_8$	mixed [c]	7.8	152	100
Chymotrypsin-treated membranes	$C_{12}E_8$	Mops	6.6	159	115

[a] Results from three separate titrations.

[b] In these two experiments, concordance between oxidative and reductive titrations was poor; slopes are mean values.

[c] In these experiments the buffer was 10 mM acetate/10 mM Mes/10 mM Hepes, the ionic strength being adjusted to 0.05 M with NaCl.

at half the maximum peak height, and determining the ratio of the width before the wavelength of maximal absorbance, to that after (the pre-λ_{max}/post-λ_{max} ratio): this ratio was plotted as a function of the degree of reduction of the cytochrome (not shown).

Determination of the haem content of cytochrome b-561

The haem content of membrane-bound cytochrome *b*-561 was determined using an immunological assay for cytochrome protein, and the pyridine-haemochrome assay for haem. Various amounts of dialysed chromaffin-granule membrane were applied to separate tracks of an SDS-polyacrylamide slab gel; other tracks contained various amounts of purified cytochrome *b*-561. After electrophoresis, the separated proteins were transferred to a cellulose nitrate sheet, which was then washed with anti-cytochrome *b*-561 serum and ^{125}I-labelled protein A. The labelled cytochrome bands were located by autoradiography, and each band was counted for radioactivity. The counts were directly proportional to loading, up to about 0.7 μg of pure cytochrome *b*-561, permitting determination of the cytochrome content of the membranes. This was calculated to be 7.1 nmol/mg protein, assuming a molecular weight of 27000 for the cytochrome (see below). The ratio of haem/total protein in this sample was 6.53 nmol/mg, so the haem/cytochrome ratio was 0.92, assuming that chromaffin granule membrane contains no other haemoprotein [21].

Crosslinking of cytochrome b-561

The existence of oligomers of cytochrome *b*-561 within the chromaffin granule membrane was investigated by treatment with several different

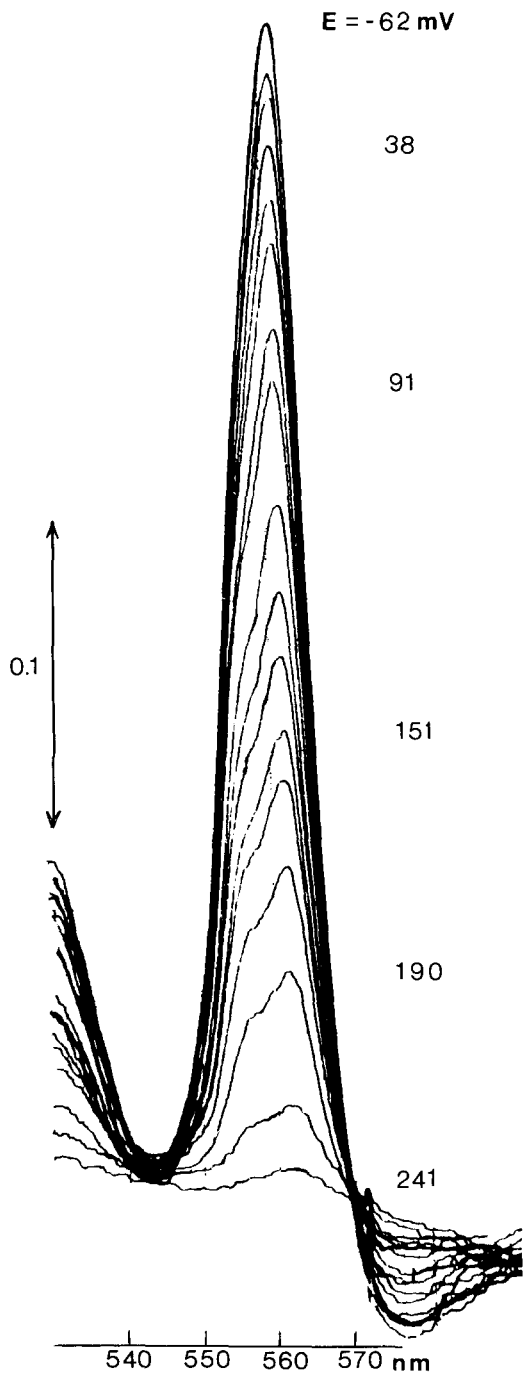

Fig. 2. Spectrophotometric redox titration of purified cytochrome *b*-561. Reduced/oxidized difference spectra were obtained after adjustment to a constant absorbance at the isosbestic wavelength, 569 nm. Protein concentration, 2.5 mg/ml; 0.05 M Mops buffer (pH 7.1), containing 0.5% (w/v) $C_{12}E_8$.

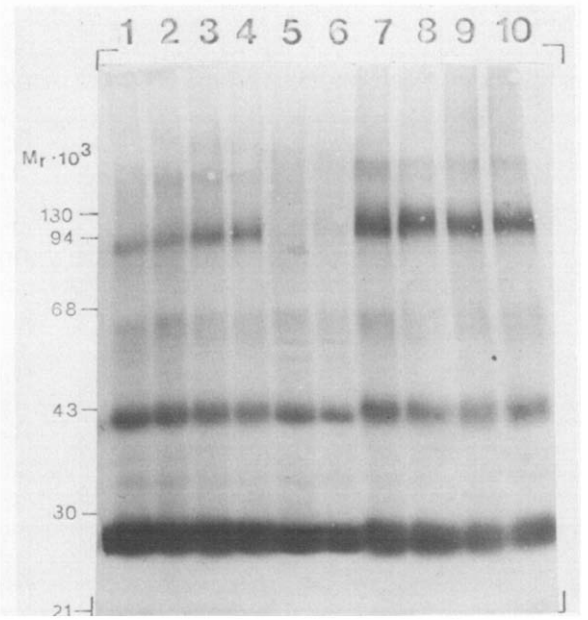

Fig. 3. Chemical crosslinking of cytochrome *b*-561 in chromaffin granule membranes. Membranes (2 mg protein/ml) were incubated in 0.05 M Bicine (pH 8.6) 30°C, for either 30 min (tracks 1, 3, 5, 7, 9) or 60 min (tracks 2, 4, 6, 8, 10). Concentrations of dimethyl suberimidate were 0.5 mg/ml (tracks 1, 2); 1.0 mg/ml (3, 4); zero (5, 6); 2.0 mg/ml (7, 8); 5.0 mg/ml (9, 10). Proteins were separated by electrophoresis and transferred to cellulose nitrate, and cytochrome *b*-561 was detected by decoration with anti-cytochrome *b*-561 and ^{125}I-labelled protein A, followed by autoradiography.

crosslinking reagents. Polyacrylamide gel electrophoresis of crosslinked membranes showed the gradual disappearance of most membrane proteins, and the formation of aggregates which failed to enter the separating gel; a number of new bands appeared, none of them intense enough to suggest its being derived from cytochrome *b*-561. The immune replica technique was therefore used to identify the products of crosslinking: the results of crosslinking with dimethyl suberimidate are shown in Fig. 3.

Aggregation of cytochrome b-561 during electrophoresis

It is noteworthy that in the immune replica shown in Fig. 3 even untreated membranes show a crossreacting band of higher molecular weight than the monomeric cytochrome. To confirm that this is related to cytochrome *b*-561, and is not simply a

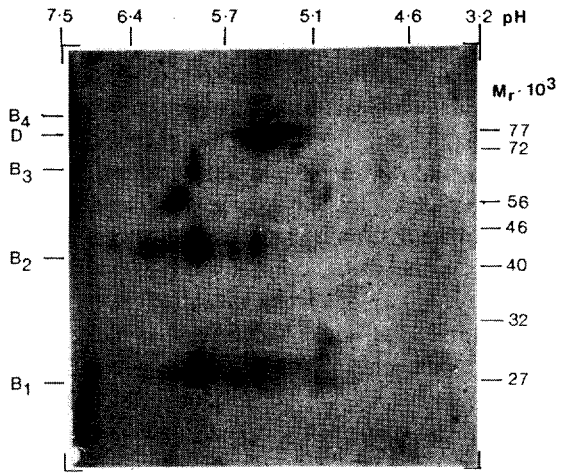

Fig. 4. Aggregation of cytochrome *b*-561 during electrophoresis. Chromaffin granule membrane proteins were separated by two-dimensional electrophoresis and transferred to cellulose nitrate. The replica was decorated with anti-cytochrome *b*-561 serum and ^{125}I-labelled protein A. B_1, B_2, B_3 and B_4 are the putative monomeric, dimeric, trimeric and tetrameric forms of cytochrome; D is ^{14}C-labelled dopamine β-hydroxylase, included as a marker. The positions of other molecular weight markers are shown on the right-hand scale.

different protein that reacts with the antiserum, chromaffin granule membranes that had not been crosslinked were subjected to two-dimensional electrophoresis, a replica made and decorated with the same antiserum. This is shown in Fig. 4; as well as the major cytochrome spot, there are at least three related species of identical p*I*, the concentrations of which decrease with increasing molecular weight. This gel was calibrated by the inclusion of chromaffin granule membrane proteins which had been labelled with 4-chloro-7-nitro[^{14}C]benzofuran (Grueninger, H.A., unpublished), some of which (e.g., dopamine β-hydroxylase) are also visible on the autoradiograph of the replica.

Aggregation of cytochrome b-561 during gel filtration

To investigate aggregation under less drastic conditions, exclusion chromatography of cytochrome *b*-561 was carried out on a column of Bio-Gel P-100 equilibrated with various eluants. The column was calibrated for each eluant system by chromatography of a mixture of standard soluble proteins. The cytochrome emerged as a single symmetric peak in each case; in $C_{12}E_8$ (0.1% w/v) or Triton X-100 (0.5%), the apparent M_r was 102000, but this was reduced to 56000 by the inclusion of 5 M urea.

Discussion

The Nernst plot for redox titration of purified cytochrome *b*-561 (Fig. 1A) differs from that expected for a simple cytochrome in having a somewhat sigmoid shape, and a slope which is greater than the 60 mV predicted by the Nernst equation. Similar plots were obtained with chromaffin granule membranes, in the presence or absence of nonionic detergent; these also have a slope greater than 60 mV, but the sigmoidicity is much less marked, and only apparent at the extremes of the plots. This anomalous behaviour might be explained in a number of ways: (1) There are two or more molecular types of cytochrome *b*-561, with different midpoint redox potentials. (2) Cytochrome *b*-561 has more than one possible environment within the membrane, so that there are two or more pools of cytochrome, structurally identical but with different midpoint redox potentials. (3) There are two or more haems per polypeptide, which either have different midpoint potentials, or show interaction during reduction and oxidation. (4) Cytochrome *b*-561 exists as an oligomer within the membrane, and there is interaction between electron-accepting centres on different polypeptide chains.

There is one previously reported redox titration of cytochrome *b*-561, carried out with dialysed chromaffin granule membranes [13]. The Nernst plot had a slightly sigmoid shape and this was taken to indicate heterogeneity of the cytochrome. The apparent midpoint potential was +140 mV. The differences from the data presented in Table I may be due to differences in temperature, or buffer: the sigmoid shape of the plots was more apparent in Mops buffer than in others. As shown in Table I, the midpoint potential of cytochrome *b*-561, whether soluble or membrane-bound, is quite sensitive to the buffer used in titration, although with each buffer, it is relatively independent of the pH. This appears not to be an effect of ionic strength,

as in the experiments in which a single buffer was used, the ionic strength was not widely varied, being close to the buffer concentration (0.05 M); in other experiments, a mixture of buffers was used, and the ionic strength adjusted to 0.05 M by addition of NaCl; here too, the variations in midpoint potential, over the pH range 5.1–7.8, were small.

The data of Fig. 1A can be fitted by assuming two molecular species of cytochrome, present in the ratio 55 : 45 and with midpoint potentials 170 and 70 mV; however, there is little evidence for such extensive structural heterogeneity. Purified cytochrome *b*-561 shows a single major component on one- or two-dimensional polyacrylamide gel electrophoresis [5], and although electrophoresis of whole membranes reveals two or even three closely migrating bands in this region [7,22], that of lowest molecular weight greatly exceeds the others in concentration. These proteins all react with the antiserum raised against cytochrome *b*-561, but the sensitivity of the immune-replica technique tends to over-estimate the proportion of the higher molecular weight components (Ref. 22 and Fig. 4).

Further evidence against heterogeneity in the redox centres comes from analysis of spectral peak widths at various stages of reduction. The pre-λ_{max}/post-λ_{max} ratio is constant throughout the titration for both purified and membrane-bound cytochrome, which strongly suggests that only one type of haem is involved. The existence of two pools of cytochrome in different environments also seems unlikely, as isolation from the membrane or detergent-treatment of the cytochrome have little effect on its redox behaviour.

The value of the haem/protein ratio depends on the molecular weight assumed for the monomeric cytochrome; if this is as low as 22000 (see below) the ratio becomes 0.75, rather than the value of 0.92 calculated if the molecular weight is 27000, as suggested by SDS-polyacrylamide gel electrophoresis. However, this strengthens the evidence against the presence of more than one redox centre in each polypeptide. It seems most likely that there is only one haem per molecule, and that the chromaffin granule membrane contains some apocytochrome.

These considerations lead us to favour the fourth explanation of the unusual Nernst plots; that the large slope is produced by negative cooperativity between haems on separate but identical polypeptide chains, within an oligomer of cytochrome *b*-561. Such interactions have been discussed elsewhere [23], and the data in Fig. 1 can be fitted quite well making such an assumption.

Some evidence for the existence of an oligomer comes from crosslinking studies, the interpretation of which is, however, complicated by some discrepancies in the apparent molecular weights of the crosslinked species. Treatment of chromaffin granule membranes with dimethyl suberimidate produces a derivative of apparent M_r 95000–100000 (Fig. 3); since the monomeric species has an apparent M_r of 27000 on this gel, the crosslinked product could be a tetramer. However, a band of apparent M_r 43000 is detectable even without treatment with crosslinker. This could, of course, be a protein unrelated to cytochrome *b*-561, but which reacts with the antiserum; but there are good reasons for believing that it, too, is a derivative of the cytochrome. Fig. 4 shows a replica of two-dimensional gel of membranes which had not been treated with crosslinker. The same protein of M_r 43000 is visible: its isoelectric point of 6.0 is identical with that of the monomeric cytochrome, and it is, like the monomer, accompanied by two minor components, of equal molecular weight but lower p*I* (5.75 and 5.5). These are the minor cytochrome variants previously revealed by haem-staining [5]. A further piece of evidence (not shown) is that if chromaffin granule membranes are dissolved in electrophoresis sample buffer and briefly heated (5 min at 100°C) before electrophoresis, cytochrome *b*-561 irreversibly aggregates and fails to enter the separating gel. No other protein is affected, but heat treatment causes the disappearance of both the major cytochrome band and that of 43000, from an immune replica with anti-cytochrome serum.

As well as this component, cytochrome oligomers of apparent M_r 64000 and 88000 are visible in the two-dimensional gel (Fig. 4). This suggests a probable monomer molecular weight of 22000; however, the major spot on the same gel has an apparent M_r of 27000. There is considerable disagreement in the literature over the molecular weight of cytochrome *b*-561. Abbs and Phillips [7] suggested a value of 22000 for chro-

momembrin B (subsequently shown to be identical with cytochrome *b*-561), as this protein had electrophoretic mobility between those of γ-globulin L-chain, and soybean trypsin inhibitor. The careful electrophoretic analysis of Duong and Fleming [9], in gels of several different concentrations, suggested a value of 30000; in our own gradient gels (e.g., Fig. 3) an apparent M_r of 27000 is obtained. From amino-acid analysis, Flatmark and Grønberg [10] calculated a value of 20500. We suggest that the monomeric cytochrome may migrate anomalously in SDS-polyacrylamide gel electrophoresis, depending on the precise conditions of the gel, and that its true molecular weight is close to 22000.

If so, the major species produced by crosslinking with dimethyl suberimidate (Fig. 3) and other crosslinkers (not shown) may not be a tetramer, but a larger aggregate. However, there is little evidence for the formation of trimers, or other intermediates other than dimers.

Could this species arise through the crosslinking of cytochrome *b*-561 to another protein? Dopamine β-hydroxylase can be crosslinked by soluble imidates [24], but the high-molecular-weight band in Fig. 3 does not react with antiserum raised against dopamine β-hydroxylase (not shown). It is not possible to exclude crosslinking to other species, but a similar band is seen upon crosslinking purified cytochrome *b*-561, suggesting that it is a homopolymer.

The tendency of cytochrome *b*-561 to oligomerize is also demonstrated by the results of exclusion chromatography in detergent solutions, although molecular weights determined by this method have to be treated with some caution. The columns were calibrated with soluble, globular proteins in the same detergents; the cytochrome has been shown to bind up to 50 mol Triton per polypeptide chain [10]. The molecular weight of 56000 determined in the presence of high concentrations of urea and Triton is quite close to that of 53000 for the detergent-protein complex, which was estimated by sedimentation studies [10].

Cytochrome *b*-561 is a transmembrane protein, with a domain on the cytoplasmic face of the granule membrane, which is susceptible to proteolysis [7]: this domain contains the major antigenic determinants [22]. Degradation with chymotrypsin reduces the apparent molecular weight by about 4000 leaving a fragment which is protected by the membrane, and which contains the haem. Crosslinking of the cytochrome may occur through the extramembrane domain, as the most extensive crosslinking was obtained with hydrophilic reagents such as dimethyl suberimidate: there was no detectable crosslinking by the hydrophobic reagent 4-fluoro-3-nitrophenyl azide. This presumably reflects the accessibility of reactive groups in the protein, as the slope of the Nernst plot is unaffected by proteolysis of the cytochrome, suggesting that interaction between polypeptide chains still occurs; the midpoint potential is significantly increased.

The results obtained suggest that the midpoint potential of cytochrome *b*-561 is relatively pH-independent in the range pH 6–8. At lower pH values membrane aggregation becomes a serious problem, and it is not possible to accurately estimate the midpoint potential at pH 5.5, the intragranular pH; nonetheless, this is clearly in the range 130–150 mV (Table I). Comparison of the values obtained over a wide range of pH values, with a mixture of buffers at constant ionic strength, suggest an upward trend as the pH is decreased (with the expection of the value at pH 5.2, which is unreliable for technical reasons). This may indicate that ionization of a group with $pK_a \leqslant 6$ in the reduced form affects the midpoint potential, but this cannot be confirmed unless methods can be devised of maintaining the cytochrome in solution at low pH, during the long times needed for deoxygenation and titration.

We have examined the effect of membrane energization on the midpoint redox potential of the cytochrome in resealed chromaffin granule 'ghosts' (unpublished results). In presence of MgATP, these 'ghosts' develop a transmembrane pH gradient and/or a membrane potential [25] which, if the midpoint potential of the cytochrome were affected, would alter the ratio of reduced/oxidized forms. We have detected no such effect, either in the presence of CNS^-, to maximize the pH gradient, or NH_3, to maximize the potential.

Similarly, none of the methods used to investigate the oligomeric state of the cytochrome has given unequivocal answers, and this problem may be best investigated by radiation inactivation anal-

ysis of the cytochrome within the granule membrane, together with further protein chemical studies.

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