Properties of the Iron–Sulphur Proteins of the Benzene Dioxygenase System from *Pseudomonas putida*

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A purification procedure was developed to stabilize the iron–sulphur proteins of the benzene dioxygenase system from *Pseudomonas putida*. The intermediate electron-carrying protein has a mol.wt. of 12300 and possesses one (2Fe–2S*) cluster, whereas the terminal dioxygenase has a mol.wt. of 215300 and possesses two (2Fe–2S*) clusters. The order and stoichiometry of electron transfer and of the whole system are described.

A strain of *Pseudomonas putida*, grown with benzene as the sole carbon source, has been shown to contain a soluble enzyme system that converts benzene into *cis*-benzene glycol (*cis*-1,2-dihydroxycyclohexa-3,5-diene) in the presence of NADH and Fe²⁺ (Axcell & Geary, 1975). The system comprised a flavoprotein and two iron–sulphur proteins. Similar systems have been reported for toluene oxidation (Yeh *et al.*, 1977) and pyrazon (5-amino-4-chloro-2-phenyl-2H-pyridazin-3-one) oxidation (Sauber *et al.*, 1977).

Purification procedures that we have reported previously (Axcell & Geary, 1975), although separating the components of the system, were subsequently found to cause some degradation of these components with respect to their contents of iron and 'inorganic' sulphur (S*); the sulphur atoms in the iron–sulphur cluster, and not the sulphur atoms from the cysteine residues through which the iron–sulphur cluster is bound to the protein). A modified purification scheme has now been developed that minimizes this degradation, thus permitting the characterization of the two iron–sulphur proteins of the benzene dioxygenase system, which is reported in the present paper.

Experimental

Polyacrylamide-gel electrophoresis

Polyacrylamide-gel electrophoresis was carried out at room temperature (20°C) with a current of 5 mA per tube (Shandon Scientific Co., London N.W.10, U.K.): 7% acrylamide gels were prepared in glass tubes (0.7 cm internal diam. × 8.5 cm) by the method of Blattler *et al.* (1972) with 10 mM-sodium phosphate buffer at pH 7, 8 and 9. The same buffer was used in both buffer reservoirs and the protein sample (50 µg) was applied directly to the top of the running gel in 1 mM-sodium phosphate buffer, containing sucrose

(12%, w/v), at the same pH. Electrophoresis was performed for the time stated for each protein and gels were stained with Amido Black (George T. Gurr, London N.W.9, U.K.) as described previously (Axcell & Geary, 1973).

Determination of iron and 'inorganic' sulphur (S*)

Samples of each protein were dialysed for the minimum time against 10 mM-KHCO₃ buffer, pH 8.5, to remove phosphate, which interferes with the determination of sulphur. 'Inorganic' sulphur was determined by the method of Lovenberg *et al.* (1963) as modified by the procedure of Suhara *et al.* (1975). A solution of Na₂S was prepared to contain approx. 30 mM-Na₂S in water, pH 8.5 (with KOH), and standardized by iodimetric titration by the arsenite method (Vogel, 1951). A linear calibration curve using this solution was prepared from 0–200 nmol of Na₂S at the same time as determinations were carried out on the protein (up to five determinations on each sample). Iron was determined by the method of Zabinski *et al.* (1972), volumes being increased 3-fold for ease of handling. All glassware was washed by soaking for 48 h in 2 M-HCl (AnalaR grade) followed by exhaustive washing in glass-distilled water. A standard solution of ferrous ammonium sulphate containing 75 nmol of Fe per 1.2 ml was prepared in glass-distilled water, and dilutions of this standard were used to prepare a linear calibration curve from 0 to 75 nmol of Fe at the same time as determinations were carried out on the protein (up to three determinations on each sample). Reagents were prepared in glass-distilled water and measurements were made against reagent blanks for both sulphur and iron determinations, by using the diffusate from the protein solutions.

Identification of the type of iron–sulphur cluster

Extrusion of the iron–sulphur clusters from both

proteins was performed by the method of Que *et al.* (1975). The proteins were dialysed for 6h against 20mM-Tris buffer adjusted to pH8.6 with HCl, and to these solutions (cold, but not in ice) was added 80% (v/v) dimethyl sulphoxide (Aldrich Chemical Co., Milwaukee, WI, U.S.A.; 'Spectrophotometric' grade). The solution was placed in a 3ml spectrophotometer cuvette with a 1cm light-path that was fitted with a septum injection port and a side arm closed by a stopcock. This cuvette was attached to an apparatus similar to that described by Stombaugh *et al.* (1976), enabling the contents to be made anaerobic by alternate evacuation and flushing with O₂-free N₂. An excess of benzenethiol (Aldrich 'Analysed' grade) in 10 μ l of anaerobic dimethyl sulphoxide was added to the anaerobic protein solution by injection through the septum port and the reaction was followed spectrophotometrically at room temperature.

Purification of benzene dioxygenase

All stages in the modified purification were carried out under an N₂ atmosphere at 4°C in 25mM-potassium phosphate buffer, pH7.3, containing 0.1mM-dithiothreitol and saturated with N₂ at atmospheric pressure (buffer I). For column chromatography the fraction collector was enclosed in a Perspex box purged continuously with N₂ and buffer reservoirs were blanketed with N₂. Direct light was excluded with aluminium foil.

An (NH₄)₂SO₄-precipitated protein fraction (0–50% saturation) was prepared from benzene-grown cells in buffer I by the method described previously (Axcell & Geary, 1973). After dialysis for 18h against 2 litres of buffer I the solution was applied to the top of a column (4cm² × 30cm) of Whatman DE-52 DEAE-cellulose (Whatman Biochemicals, Maidstone, Kent, U.K.) equilibrated in buffer I. Proteins were eluted with a 0–6% (w/v) linear gradient of KCl in 500ml of buffer I, fractions (9ml) being collected at 15min intervals. Assays for components of the benzene dioxygenase system were carried out as previously described (Axcell & Geary, 1973, 1975). Fraction A (containing iron-sulphur protein, A1; flavoprotein, A2; and pyrocatechase) was located in tubes 20–29 and fraction B (containing iron-sulphur protein B and *cis*-benzene glycol dehydrogenase) was located in tubes 30–39. The contents of these tubes were pooled separately and concentrated to 6–8ml by using an Amicon PM-10 membrane filter (Amicon, High Wycombe, Bucks., U.K.). [It will be shown below that protein B passes electrons from protein A2 (reduced by NADH) to protein A1, which is the dioxygenase enzyme.]

Fraction A. Three portions of protein obtained as described above were combined and further concentrated to 10–15ml (Amicon PM-10) before application to the top of a column of Bio-Gel A 0.5m

(7cm² × 80cm). (Bio-Rad Laboratories, Richmond, CA, U.S.A.) equilibrated in buffer I and eluted with the same buffer. Fractions (9ml) were collected at 30min intervals. Protein A1 was identified by its spectrum (Fig. 1). Two contaminating proteins having λ_{max} 420 and 410nm were eluted on either side of protein A1, which is characterized by its absorption maximum at 450nm. Fractions 20–23, containing the minimum amount of these contaminants, were pooled and concentrated to 5–6ml before application to a column (3cm² × 80cm) of Sephadex G-150 (superfine grade) (Pharmacia Fine Chemicals AB, Uppsala 1, Sweden) in buffer I. Proteins were eluted from this column with the same buffer. Fractions (4ml) were collected at 30min intervals and protein A1 was again identified by its spectrum. Fractions 29–32 were pooled, thereby further eliminating contaminating proteins, and concentrated to 5–6ml. This step was repeated and the final concentrate was stored frozen in 0.5ml portions. The protein was shown to be virtually free of contaminating proteins at this stage by polyacrylamide-gel electrophoresis (Fig. 2a) and it sedimented as a single peak in the analytical ultracentrifuge. A fraction containing protein A2 and pyrocatechase was located in tubes 25–27 from the Bio-Gel A 0.5m column and the

Fig. 1. Spectra of protein A1, oxidized (a) and after enzymic reduction by titration with NADH (b). Conditions are described in the text.

Fig. 2. Polyacrylamide-gel electrophoresis of proteins A1 and B

(a) Protein A1 after 2.5 h at pH 8; (b) protein B after 1.75 h at pH 7. Conditions are described in the text.

contents of these tubes were pooled and concentrated to 5–6 ml before freezing in 0.5 ml portions for use in reactions described below.

Fraction B. Fraction B was similarly purified through the columns described for fraction A, KCl (100 mM) being included in all buffers to stabilize the iron-sulphur protein. Fractions were again identified by their spectra, the spectrum of pure protein B having an absorption maximum at 460 nm (Fig. 3). Fractions 42–44 were pooled from the Bio-Gel A 0.5 m column and concentrated (Amicon UM-2) to 5–6 ml before application to the Sephadex G-150 (superfine grade) column. Fractions 52–54 from this column were pooled and concentrated to 5–6 ml and frozen in 0.5 ml portions. The protein at this stage yielded a single major band on gel electrophoresis, the band being rather diffuse (Fig. 2b). It appeared to be homogeneous in the analytical ultracentrifuge and it was eluted as a sharp single peak from Sephadex G-150 (superfine grade) (calibrated column for molecular weight determination). Fractions 23–30 from the Bio-Gel A 0.5 m column contained *cis*-benzene glycol dehydrogenase and these were pooled and concentrated (Amicon PM-10) to 10–12 ml and stored frozen in 1 ml portions.

Fig. 3. Spectra of protein B, oxidized (a) and after enzymic reduction by titration with NADH (b). Conditions are described in the text.

Spectrophotometry

All spectrophotometric measurements were made by using a Pye-Unicam SP. 1800 double-beam recording spectrophotometer. Samples were contained in quartz cuvettes of 1 and 3 ml volume, with a light path of 1 cm. Temperature was controlled by a Churchill circulating-water heater (Churchill Instrument Co., Perivale, Middx., U.K.).

Stoichiometry of electron transfer

Bleaching of the characteristic absorption maxima at 450 nm (protein A1) and 460 nm (protein B) by NADH in the presence of catalytic amounts of protein A2 (for protein B) and proteins A2+B (for protein A1), at 30°C, was used to demonstrate the number of electrons carried by each protein.

Protein A1. A solution containing 12 mg of protein A1 (0.056 μ mol), 0.35 mg of protein B (0.99 atom of Fe/molecule) and 1.3 mg of protein A2 in a total volume of 2.8 ml of 25 mM-potassium phosphate buffer, pH 7.3, was rendered anaerobic in the special cuvette described above. Portions (30 μ l) of an anaerobic solution of NADH, the concentration of which was determined as 0.046 μ mol/100 μ l (from the A_{340} by using an absorption coefficient of 6.2×10^3 litre $\cdot$ mol $^{-1} \cdot$ cm $^{-1}$), were added by injection through the septum port. A graph was plotted of A_{450} (corrected for the change in volume) against NADH added (μ l). A line projected from the initial slope of this graph represents the utilization of NADH under the initial conditions where protein

A1 (oxidized) is in excess and proteins A2 and B are both in the oxidized form. The oxidation of small additions of NADH will therefore be virtually complete.

Protein B. The solution of protein B contained 3.3 mg of protein/ml. Fe was estimated as 1.46 atoms per molecule in this preparation, yielding a value of 2.4 mg of (2Fe-2S*) protein B/ml, assuming the holoenzyme to be diluted with protein of a similar molecular weight (see below). The reaction mixture contained 0.55 ml of this solution equivalent to 0.107 μ mol of (2Fe-2S*) protein B, and 1.3 mg of protein A2 in a total volume of 2.8 ml. The titration was carried out as above by adding portions (20 μ l) of NADH solution (0.045 μ mol/100 μ l) and recording the A_{460} .

Overall stoichiometry

The overall stoichiometry of the conversion of benzene into *cis*-benzene glycol was determined by using the oxygen-electrode apparatus described

(20 μ l) of FAD, 4.3 mg (200 μ l) of protein A1, 0.72 μ mol (20 μ l) of FeSO₄·7H₂O, 0.165 mg (50 μ l) of protein B and 1.423 μ mol (150 μ l) of NADH. The reaction was initiated after equilibration by the addition of a solution of benzene (30 μ l; 11.25 mm) in 25 mM-potassium phosphate buffer, pH 7.3. O₂ consumption was recorded and corrected for endogenous respiration. The cell was then opened to re-admit O₂ thereby diluting the *cis*-benzene glycol formed with the volume of buffer excluded from the cell by the additions. The cell was then closed after re-aeration of the contents and the amount of *cis*-benzene glycol present in the stirred volume was calculated from the amount of benzene added, corrected for dilution. Purified *cis*-benzene glycol dehydrogenase (1 mg; 50 μ l) was then injected causing a further consumption of O₂ as the *cis*-benzene glycol was converted into catechol and then into *cis,cis*-muconic acid (Ornston & Stanier, 1966) by the pyrocatechase present in protein fraction A2 (eqns. 1 and 2).

previously (Rank Bros., Bottisham, Cambridge, U.K.; Axcell & Geary, 1975). The temperature was controlled at 20°C by using a Tecam circulating-water bath with dip-cooler [Techne (Cambridge), Duxford, Cambridge, U.K.]. The cell volume for gaseous exchange was adjusted to 3 ml and the total oxygen content of this volume of buffer at 20°C was 0.86 μ mol (Glasstone, 1946). Additions were made by injection to 3 ml of 25 mM-potassium phosphate buffer, pH 7.3, after establishment of a full-scale recorder deflection of 100 divisions for 0.86 μ mol of O₂. The solution that was displaced from the stirred volume by these additions was retained in the hollow stopper of the cell above the injection port and allowed for in calculations.

NADH/O₂. To 3 ml of buffer was added: 1.3 mg (100 μ l) of protein A2, 50 nmol (20 μ l) of FAD, 4.3 mg (200 μ l) of protein A1, 0.72 μ mol (20 μ l) of FeSO₄·7H₂O, 0.165 mg (50 μ l) of protein B and 1.125 μ mol (100 μ l) of benzene in 25 mM-potassium phosphate buffer, pH 7.3. The reaction was initiated after equilibration by the addition of portions of a solution of NADH (10–40 μ l) containing 9.48 mM-NADH in 25 mM-potassium phosphate buffer, pH 7.3, and oxygen consumption was recorded and corrected for endogenous respiration.

Benzene/O₂/*cis*-benzene glycol. To 3 ml of buffer was added: 1.3 mg (100 μ l) of protein A2, 50 nmol

Ultracentrifuge measurements

Ultracentrifuge measurements were made by using a Beckman model E analytical ultracentrifuge equipped with schlieren and interference optics (Beckman-RIIC, Glenrothes, Fife, Scotland, U.K.). Sedimentation-velocity data were determined at 20°C and 56000 rev./min by using a double-sector cell fitted with sapphire windows. The diffusion coefficient was calculated by the area-height method (Schachman, 1957) from data obtained at 20°C and 6000 rev./min by using the above cell fitted with a double-sector capillary synthetic-boundary centre-piece. Molecular weights were determined by the meniscus-depletion method of Yphantis (1964) in a double-sector interference cell fitted with sapphire windows.

Protein A1. A solution of protein A1 (1–2 mg/ml) was dialysed against buffer I for 18 h at 4°C and centrifuged at 13000–14000 rev./min. Photographs were taken after equilibrium had been established at the chosen speed (36–72 h) by using interference optics. The diffusate was used as the reference solution.

Protein B. A solution containing protein B (1–2 mg/ml) was similarly dialysed against buffer I containing 100 mM-KCl. Equilibrium was attained at speeds from 48000 to 52000 rev./min. The diffusate was used as the reference solution. All measurements were

made on glass photographic plates (Kodak metallographic plates; Eastman Kodak Co., Rochester, NY, U.S.A.) with a Projectorscope travelling microscope (Precision Grinding, Mitcham, Surrey, U.K.).

Measurement of protein concentration

Protein was measured by the modified biuret method of Layne (1957) with a freshly dried sample of bovine serum albumin as the standard.

Reagents

Reagents for polyacrylamide-gel electrophoresis were obtained from Kodak, Kirkby, Liverpool, U.K. *cis*-Benzene glycol was prepared by the method of Nakajima *et al.* (1956). All other reagents were obtained from BDH, Poole, Dorset, U.K. (AnalaR grade) and Sigma (London) Chemical Co., Kingston upon Thames, Surrey, U.K., except where stated in the text. Components of the benzene dioxygenase system required for assays were obtained during the purification of the proteins under study, as stated in the text.

Results

Determination of molecular weights

The molecular weight of protein A1 was calculated to be 215 300 (average of four values) using a value of 0.747 for $\bar{v}$ (Svedberg & Pederson, 1940). This value for $\bar{v}$ is for average proteins and assumes that the contribution from the Fe-S* cluster is negligible compared with the size of the protein. The molecular weight of protein B was calculated as 12600 from photographs taken at 30h. The value for $\bar{v}$ was estimated as 0.711 from data given by Devanathan *et al.* (1969) for variations in $\bar{v}$ for small iron-sulphur proteins. During runs longer than 40h material was seen to diffuse back into the meniscus as the protein became degraded. The molecular weight was therefore confirmed by gel filtration on a column (1.75 cm² × 85 cm) of Sephadex G-150 (superfine grade) equilibrated and eluted with buffer I containing 100 mM-KCl (Andrews, 1964). The column was calibrated with proteins of known molecular weight (Weber & Osborn, 1969), the elution volume of protein B corresponding to a mol.wt. of 12000 (Fig. 4). An average value of 12300 for the molecular weight of protein B was used in subsequent calculations.

Sedimentation and diffusion coefficients

Protein A1 (8 mg/ml) was dialysed against buffer I and the sedimentation coefficient ($s_{20,w}$) was determined as 9.85S. By using the same solution the diffusion coefficient ($D_{20,w}$) was found to be 4.06×10^{-7} cm² · s⁻¹. Protein B (12 mg/ml) was dialysed against buffer I, which contained 100 mM-KCl, and $s_{20,w}$ and $D_{20,w}$ were determined as 1.71S and

Fig. 4. Determination of the molecular weight of protein B by gel filtration on a column of Sephadex G-150 (superfine grade)

For details see the text.

10.85×10^{-7} cm² · s⁻¹ respectively. In all cases the diffusate was used as the reference buffer solution.

Iron and 'inorganic' sulphur content

Both proteins of the benzene dioxygenase system containing iron and 'inorganic' sulphur have spectra that are typical of proteins containing (2Fe-2S*) or (4Fe-4S*) clusters for electron transfer (Holm, 1975) (Figs. 1 and 3).

Protein A1 was found to contain 4.1 ± 0.2 (mean $\pm$ s.d.) atoms of iron and 4.1 ± 0.4 (mean $\pm$ s.d.) atoms of sulphur per molecule of mol.wt. 215 300 (average of four determinations), yielding a nearest integral value of 4 atoms of each per molecule. Protein B routinely contained between 1 and 1.5 atoms of both iron and sulphur per molecule of mol.wt. 12300. The amounts of each element however were always equivalent within the experimental error (e.g. 0.99 Fe; 1.03 S*). Since this protein is labile and since proteins with less than 2 iron atoms per molecule have been shown not to contain S* (Tsibris & Woody, 1970), it is presumed that protein B consists of an iron-sulphur protein with a minimum of 2 atoms each of iron and S* per molecule, the remaining protein being apoenzyme or a contaminating protein with similar properties of size and charge. Yeh *et al.* (1977) have also indicated that the equivalent protein from the toluene dioxygenase system may contain 2 iron and 2 S* atoms per molecule.

Iron-sulphur clusters of proteins A1 and B

In each case the protein was soluble at the concentrations used in 80% (v/v) dimethyl sulphoxide and showed a shift in the spectrum from 450–460 nm to

approx. 430nm, indicating unfolding of the polypeptide chain. On addition of an excess of benzenethiol the extrusion reactions proceeded smoothly over a period of 30min to yield, for protein B, a compound having $\lambda_{\max}$ at 490nm with a spectrum identical with that of the model compound $[\text{Fe}_2\text{S}_2(\text{SPh})_4]^{2-}$ ($\text{Ph}=\text{C}_6\text{H}_5$), and with that of the extrusion compound derived from *Spirulina maxima* ferredoxin with benzenethiol (Que *et al.*, 1975), indicating protein B to have a $(2\text{Fe}-2\text{S}^*)$ cluster. Protein A1 yielded a compound with a spectrum having $\lambda_{\max}$ at 474nm, which is intermediate between the data given for $[\text{Fe}_4\text{S}_4(\text{SPh})_4]^{2-}$ ($\lambda_{\max}$ 458nm) and $[\text{Fe}_2\text{S}_2(\text{SPh})_4]^{2-}$ ($\lambda_{\max}$ 490nm), but closely resembles the spectrum of the extrusion product from $(2\text{Fe}-2\text{S}^*)$ adrenodoxin (Erbes *et al.*, 1975). (Increasing the concentration of benzenethiol in an attempt to prevent the possible formation of the tetramer compound by dimer-tetramer interconversion resulted in a precipitate forming and loss of resolution of the peak at 474nm.) Spectra thus obtained from proteins B and A1 were stable over a further period of 60min (Fig. 5).

Since protein B has been shown to contain more than 1 iron and 1 S^* atom per molecule of mol.wt. 12300, it is concluded that preparations contained a mixture of $(2\text{Fe}-2\text{S}^*)$ protein B and either the apoenzyme lacking the iron-sulphur cluster or a contaminating protein with similar properties of charge and molecular weight. Protein A1, having 4 iron and 4 S^* atoms could either contain one $(4\text{Fe}-4\text{S}^*)$ cluster or two $(2\text{Fe}-2\text{S}^*)$ clusters per molecule. The latter state is indicated by the cluster-extrusion data and is borne out by the following experiments.

Spectrophotometric determinations

Order of components in the electron-transfer chain. The order in which electrons are transferred from NADH through the components of the benzene dioxygenase system was established by following the bleaching of the A_{450} of protein A1 caused by reduction of the iron-sulphur cluster. All proteins were dialysed against 25mM-potassium phosphate buffer, pH7.3, to remove dithiothreitol and to ensure that they were in the fully oxidized state. Reaction mixtures were made up to a total of 3ml with the same buffer in a 3ml cuvette and reactions were carried out at 30°C. No reduction of protein A1 occurred when 13mg of protein A1 (approx. $0.06\mu\text{mol}$) was incubated with $1\mu\text{mol}$ of NADH alone, or in combination with either 0.33mg of protein B or 1.3mg of protein A2. In the presence of all components, however, the reduction of protein A1 proceeded to completion within 2min, yielding the same spectrum as that produced in the presence of sodium dithionite (Fig. 1). Since flavoprotein A2 has been shown to transfer electrons from NADH to the artificial electron acceptor dichlorophenol-indophenol (Axcell &

Fig. 5. Spectra of iron-sulphur cluster-extrusion products from proteins A1 (----) and B (—) in 80% (v/v) dimethyl sulphoxide

For protein A1 the reaction mixture contained 9mg of protein ($0.04\mu\text{mol}$) and $5\mu\text{mol}$ of benzenethiol in a total volume of 3ml of 80% (v/v) dimethyl sulphoxide. The spectrum was recorded after 60min at 20°C. The aqueous component of the mixture was 20mM-Tris/HCl, pH8.6. For protein B the reaction mixture contained 2mg of protein ($0.162\mu\text{mol}$) and $6\mu\text{mol}$ of benzenethiol. Other conditions were as for protein A1.

Geary, 1975), the fact that protein A1 is not reduced unless protein B is present confirms the sequence:

$\text{NADH} \rightarrow \text{flavoprotein A2} \rightarrow \text{iron-sulphur protein B} \rightarrow \text{iron-sulphur protein A1} \rightarrow \text{benzene}$

Protein A1 is therefore confirmed as the dioxygenase enzyme.

Stoichiometry of electron transfer

Protein solutions that had been dialysed against 25mM-potassium phosphate buffer, pH7.3, as des-

Fig. 6. Titration of protein A1 with NADH in the presence of proteins A2 and B (a) and titration of protein B with NADH in the presence of protein A2 (b). For details see the text.

Table 1. Stoichiometry of the benzene dioxygenase reaction

Demonstration of equivalence between NADH and O_2 . For details see the text. The results are the average of two determinations at each concentration.

NADH present in the stirred volume of 3 ml (μmol)	O_2 used, corrected for endogenous respiration (μmol)	Molar ratio of O_2 /NADH
0.095	0.090	0.95
0.188	0.170	0.90
0.282	0.265	0.94
0.374	0.353	0.94

Table 2. Stoichiometry of the benzene dioxygenase reaction

Demonstration of equivalence between benzene, O_2 and *cis*-benzene glycol. For details see the text. Results are given for duplicate determinations.

Benzene present in the stirred volume of 3 ml (μmol)	First O_2 uptake, corrected for endogenous respiration (μmol)	Molar ratio of O_2 /benzene
0.334	0.348	1.04
0.334	0.344	1.03
Calculated amount of glycol in 3 ml (μmol)	Second O_2 uptake, corrected for endogenous respiration (μmol)	Molar ratio of glycol/benzene
0.278	0.275	0.99
0.278	0.286	1.03

cribed above, were used. Each solution was checked for the absence of cross-contamination by the usual assays. The fraction containing protein A2 contained pyrocatechase, but *cis*-benzene glycol dehydrogenase was absent from all components.

Protein A1. In the presence of excess NADH the total absorbance change (ΔA_{450}) was found to be 0.162 unit. Under these conditions protein B is also fully reduced and it may be calculated (from the protein concentration, iron content and oxidized and reduced spectra) that the amount of protein B present would yield a ΔA_{450} of 0.009 unit. The corrected ΔA_{450} for 0.056 μmol of protein A1 is therefore 0.153 unit, equivalent to 118 μl (0.054 μmol) of NADH (Fig. 6a).

Protein B. A total absorbance change (ΔA_{460}) of 0.093 unit was recorded for 0.107 μmol of protein B, equivalent to 119 μl (0.054 μmol) of NADH (Fig. 6b).

In each case the protein was allowed to reoxidize in air. The addition of excess of solid sodium dithionite was shown to yield the same change in absorbance as did enzymic reduction, and the same reduced spectra were obtained (Figs. 1 and 3). The results indicate that 1 mol of NADH would yield 2 mol of reduced protein B and 1 mol of reduced protein A1.

Overall stoichiometry

NADH/ O_2 . Results from oxygen-electrode experiments showed that the consumption of O_2 (μmol) was equivalent to the amount of NADH added (μmol) in the presence of an excess of benzene (*cis*-benzene glycol dehydrogenase was absent from these enzyme preparations, protein fraction A2 contained pyrocatechase) (Table 1).

Benzene/ O_2 /*cis*-benzene glycol. The initial consumption of O_2 (μmol) was shown to be equivalent to the amount of benzene added (μmol) in the presence of an excess of NADH. The second consumption of O_2 , initiated by the addition of *cis*-benzene glycol dehydrogenase in the presence of pyrocatechase, was equivalent to the calculated