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INTERACTIONS OF SUCCINATE DEHYDROGENASE WITH CYANIDE

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SUMMARY

Succinate dehydrogenase (succinate:(acceptor) oxidoreductase, EC 1.3.99.1) solubilized by KCN, differs from other soluble preparations of this enzyme for the following properties.

1. It has 16–20 nmoles of tightly bound cyanide per mg protein.

2. It contains 3.6–4.1 nmoles of peptide-bound flavin, 24–32 natoms of iron and 16–17 nmoles of acid-labile sulphide per mg protein. An alteration of the iron–sulphur prosthetic group is thus indicated.

3. Density gradient centrifugation shows two active species of mol. wt 180 000 and 140 000. By gel filtration in chaotropic agents, two components of mol. wt 180 000 and 100 000 can be isolated. The molecular weights, by gradient ultracentrifugation, of the isolated components correspond to the original values obtained for the unfractionated enzyme. It is proposed that the heavier species is a dimer stabilized by cyanide; the lighter one represents the monomeric form of the flavoprotein, which exists in a dynamic monomer–dimer equilibrium.

The mechanism of action of KCN in the dissociation of succinate dehydrogenase from the membrane was also investigated.

1. CN^- is shown to be the species acting in the process. The reaction is found to be first order with respect to CN^-.

2. KCN induces spectral modifications on soluble succinate dehydrogenase and an anomalous stability to iron: two moles of CN^- per gatom iron remain bound to protein after mild acid treatment.

3. It is concluded that CN^- binds to the iron of the membrane-bound succinate dehydrogenase. As a result of a probable conformational change, the resolution of the linkages to the membrane occurs.

INTRODUCTION

In 1951, Tsou[1] reported that upon incubation of heart muscle preparations with cyanide, the oxidation of succinate by methylene blue was slowly inhibited. This inactivation reaction was found to be irreversible, dependent on temperature and concentration of KCN, but not on pH, and to be prevented by reducing conditions.

Giuditta and Singer[2] showed that cyanide inhibits only partially the activity of the membrane-bound enzyme with phenazine methosulphate as acceptor, but not the activity of the soluble succinate dehydrogenase. They suggested the possibility that ferric iron was the reaction site of CN^-.

Contrary to this, Keilin and King[3] proposed that cyanide interrupts the contacts of succinate dehydrogenase with the other carriers of the respiratory chain supposedly through a cyanolysis of a disulphide bridge. An important finding was that of Wilson and King[4] who reported that Keilin–Hartree heart muscle preparations treated with cyanide lack succinate dehydrogenase activity and are capable of reincorporating the soluble enzyme. They interpreted this as further evidence that cyanide can break the flavoprotein linkages to the respiratory chain. Definite experimental evidence, however, was given by Wu and King[5], who showed that during treatment with KCN, succinate dehydrogenase was resolved from the membrane and became soluble. A new method for solubilizing succinate dehydrogenase from the membrane, not involving extraction with organic solvents or alkali, was thus shown.

One of the aims of the present investigation was to study the properties of succinate dehydrogenase solubilized by KCN. In addition it was attempted to discriminate between the hypotheses that have been put forward to explain the mechanism of solubilization by KCN and to establish the chemical species acting in this process.

In this paper we report the catalytic and molecular parameters of the partially purified cyanide-enzyme and provide evidence for the site of action of KCN. A further interesting finding is the preferential interaction of cyanide with the activated form of succinate dehydrogenase (Zanetti, G. and Galante, Y. M., unpublished).

Preliminary results have been published elsewhere[6–8].

MATERIALS AND METHODS

Cytochrome *c* (Type III, horse heart), aldolase (rabbit muscle), alcohol dehydrogenase (yeast), α-chymotrypsinogen A (bovine pancreas), catalase (beef liver), ovalbumin, bovine serum albumin, and riboflavin were obtained from Sigma Chemical Co.; haemoglobin was a gift of Dr L. Rossi-Bernardi. $K^{14}CN$ was from The Radiochemical Centre, Amersham (England) and from Sorin, Saluggia (Vercelli). All other chemicals were analytical grade from Merck or B.D.H.

Succinate dehydrogenase was isolated from beef heart muscle preparation and purified as previously described[6] with the following modifications which improved resolution. Incubation with 50 mM KCN (45 min at 30 °C) was performed in 0.1 M phosphate buffer (pH 7.4) and the gel filtration on a Sephadex G-200 column (100 cm × 3 cm) was effected in upward flow by means of a peristaltic pump, which gave a constant flow of 40 ml/h. The active fractions were precipitated with $(NH_4)_2SO_4$ at 65% saturation and the pellet was stored in liquid N_2. Before further use it was desalted on Sephadex G-25.

Succinate dehydrogenase activity was assayed spectrophotometrically as described previously[6], but succinate was 20 mM; when assaying the particulate enzyme 1.5 mM KCN was also present. Phenazine methosulphate was omitted when measuring 2,6-dichlorophenolindophenol reductase activity. Activation was performed in 40 mM succinate, 50 mM phosphate buffer (pH 7.6) at 25 °C. The cyanide-enzyme

was preincubated for 5 min; the particulate one for 20 min, and 1.5 mM KCN was added.

Protein was estimated, up to the enzyme solubilization step, by the biuret method[9], while at later stages the microbiuret method[10] was used. Crystalline bovine serum albumin was used as standard reference, and the correction proposed by Davis and Hatefi[11] was not applied.

Protein-bound flavin was determined fluorimetrically, with a Perkin–Elmer spectrofluorimeter Model MPF 2 A, according to Cerletti and Giordano[12]. No correction was made for the fluorescence at pH 7.

Iron was quantitatively estimated by wet ashing[13]. Labile sulphide was determined by the method of Fogo and Popowski[14], as modified by King and Morris[15].

Radioactivity was counted using a Packard-Tricarb at 80% efficiency. The counting medium contained up to 0.2 ml aqueous sample, 0.2–1.0 ml soluene (Packard) as solubilizing agent, and 15 ml of the scintillation mixture.

RESULTS

Chemical species acting in solubilization

Owing to the controversial data of the literature on the pH dependence of KCN action[1,2,5], it was felt necessary to prove beyond doubt which is the species involved in the process of solubilization of succinate dehydrogenase from the membrane: CN^- or HCN.

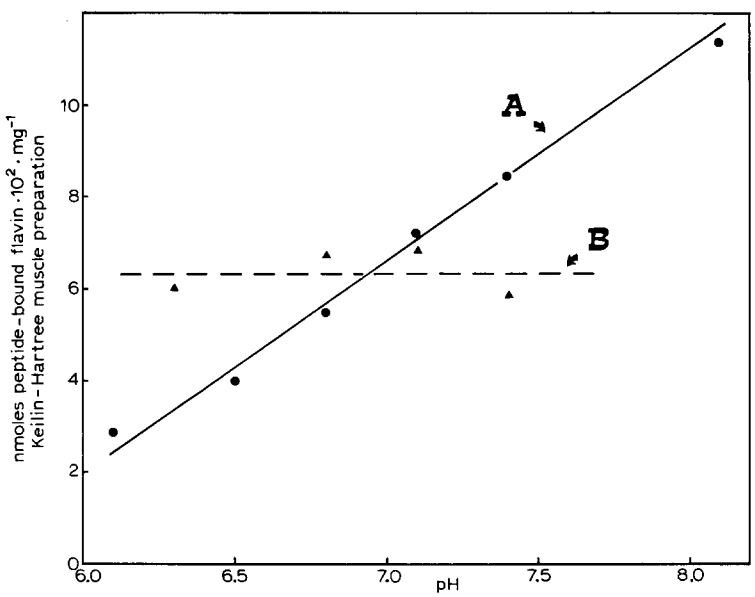

Fig. 1. Solubilization of succinate dehydrogenase as function of CN^- concentration. Curve A (●——●), KCN was 10 mM; Curve B (▲– – –▲), KCN concentrations were such as to give 0.1 mM CN^- in all the incubations, assuming a pK_a of 9.1 for HCN. Keilin–Hartree heart muscle particles (12 mg protein·ml^{-1}) were incubated with KCN in 0.1 M phosphate at 30 °C for 45 min. After centrifugation for 60 min at 32 000 × *g*, an aliquot of the supernatant was precipitated at 65% satn of $(NH_4)_2SO_4$ to remove excess cyanide. Peptide-bound flavin was determined on the precipitate.

Fig. 1 shows the typical behaviour of the reaction as a function of pH; a linear relationship is obtained at a fixed concentration of KCN (Curve A), at least in the range 5–50 mM. Curve B demonstrates the complete pH independence of the process, provided the concentration of CN^- is kept constant.

The extraction of the flavoprotein is thus a function of the concentration of CN^-.

Kinetics of solubilization

The kinetics of the cyanide reaction was studied by a more suitable test, than the time-consuming and laborious determination of the extracted protein-bound flavin. Succinate dehydrogenase loses its ability to react directly with 2,6-dichlorophenolindophenol, when in soluble form[16]. Since this property is also shown by the cyanide-enzyme, it is possible to follow the decrease of succinate: 2,6-dichlorophenolindophenol reductase activity of Keilin–Hartree heart muscle preparations, as an alternative measure of solubilization. The following observations confirmed that both parameters describe the same reaction: succinate prevents the extraction of succinate dehydrogenase as well as the inactivation of 2,6-dichlorophenolindophenol reductase activity by KCN and the half-times of both reactions are the same.

Keilin–Hartree heart muscle partides (12 mg protein·ml^{-1}) were incubated at various concentrations (10–75 mM) of KCN (pH 7.4). At various times, aliquots were fully activated with succinate and 2,6-dichlorophenolindophenol reductase activity measured. The inactivation reaction in excess of cyanide follows first-order kinetics and the rate is a linear function of the concentration of CN^-. The rate constant was calculated to be 30.6 $M^{-1}\cdot min^{-1}$.

The reaction order with respect to cyanide was determined as described by Levy *et al.*[17] and found to be one (Fig. 2).

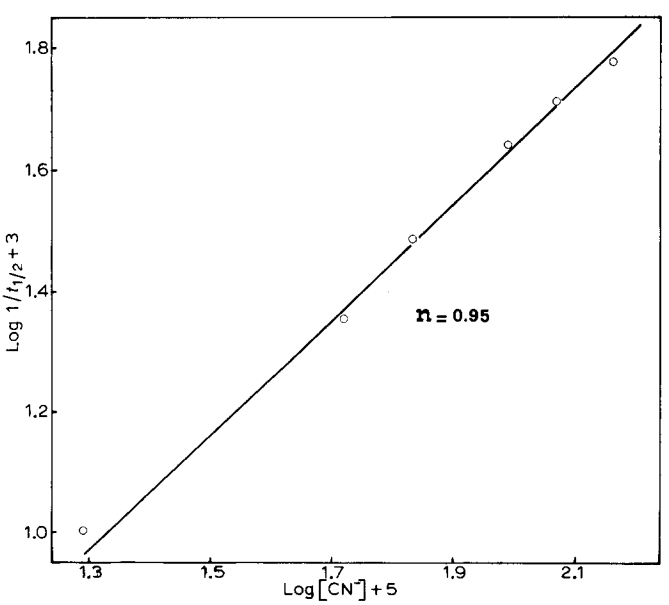

Fig. 2. Determination of the order of reaction between membrane-bound succinate dehydrogenase and CN^- with respect to CN^-. The slope of the straight line gives n as 0.95.

Catalytic properties

The partially purified preparation of succinate dehydrogenase solubilized by cyanide treatment contains one mole peptide-bound flavin per 260 000 (± 10%) g protein and has a specific activity of approximately 9 μmoles succinate oxidized·min^{-1}·mg^{-1}. This preparation is not contamined by haem iron as ascertained by spectral analysis after destroying the non-haem iron chromophore with *p*-chloromercuribenzoate.

The treatment with cyanide releases about 70% of the total peptide-bound flavin of the Keilin–Hartree heart muscle particles, but produces a considerable decrease (60%) of the total succinate dehydrogenase activity. The loss of activity should be attributed, in part, to inactivation of the enzyme due to the absence of succinate during the treatment. Indeed it is well known that succinate stabilizes the activity of succinate dehydrogenase and is required during extraction in order to have a reconstitutively active enzyme[18]. The occurrence of an additional thermal inactivation is plausible.

A specific inhibitory effect of cyanide can be excluded on the basis of experiments with succinate dehydrogenase solubilized by butanol.

Anaerobic conditions do not improve the total yield of activity solubilized from the membrane by KCN.

Cyanide-enzyme differs from other soluble succinate dehydrogenase preparations with respect to stability[19,20] and succinate activation[21]. At 0 °C in air, no loss of activity is observed for several hours and by incubation with succinate the activity is enhanced 1.4 times only.

Composition

Experiments with $K^{14}CN$, during solubilization with cyanide, showed that succinate dehydrogenase incorporates 4–6 moles cyanide per mole peptide-bound flavin, which remain strongly bound throughout purification[6]. The cyanide-enzyme contains 3.6–4.1 nmoles of peptide-bound flavin, 24–32 natoms of iron, 16–17 nmoles of acid-labile sulphide and 16–20 nmoles of cyanide per mg protein.

These values might suggest the following stoichiometry: peptide-bound flavin: Fe:labile sulphide:cyanide = 1:8:4:5. However, we observed that when all four parameters were determined on the same enzyme preparation, the amount of cyanide bound was approximately twice the difference between the non-haem iron and the labile sulphide present. Therefore, the stoichiometry based on the mean values of several assays does not account for the scattering between different preparations and may not describe the actual situation. Furthermore, the content of labile sulphide in cyanide-enzyme is always lower than that of the non-haem iron, whereas in the native or otherwise solubilized succinate dehydrogenase[11,22] the ratio is 1:1. Only Veeger and co-workers[23,24] found a lower ratio, even for the butanol-solubilized enzyme.

Binding of cyanide

Methods[25,26] known to release non-haem iron or even total iron from protein and mitochondria, were used to determine the iron content of the cyanide-enzyme. Lower values as compared to wet ashing[13], were constantly found. Since this behaviour was peculiar to succinate dehydrogenase solubilized by cyanide, it was of

TABLE I

CYANIDE AND IRON BOUND IN SUCCINATE DEHYDROGENASE AFTER ACID EXTRACTION

Values are expressed as moles (gatoms) per mole peptide-bound flavin. The iron before treatment and after treatment bound was measured by wet ashing[13]. The iron after treatment released was determined according to Doeg and Ziegler[25].

Treatment applied	*Enzyme treated*	*Cyanide*			*Iron*			*Protein bound* CN^-:*Fe*
		Before treatment	*After treatment*		*Before treatment*	*After treatment*		
			Released	*Bound*		*Released*	*Bound*	
Trichloro-	Cyanide-enzyme	6.5	1.7	4.9	8.4	—	—	—
acetic acid		4.2	0.3	3.6	6.4	3.0*	1.5	2.4
		4.2	0.4	3.4	6.4	—	1.6	2.1
Acetic acid	Cyanide-enzyme	5.2	1.7	3.7	8.4	—	2.2	1.7
+ mercapto-		4.2	0.9	2.9	6.4	4.8	1.9	1.5
acetic acid		4.2	0.9	2.7	6.4	—	1.3	2.1
	Succinate dehydrogenase, KCN treated	2.6	0.9	1.4	4.9	4.3	0.6	2.2

* Further iron is extracted by washing.

interest to follow the fate of the incorporated cyanide, during the same treatments of iron extraction. The results of such experiments are reported in Table I. It should be recalled[27] that all the iron and cyanide are released by incubation in 1 M HCl for 60 min at 100 °C, according to the method of King *et al.*[28] for non-haem iron. Two different extraction procedures were applied to the cyanide-enzyme: either a treatment with 10% trichloroacetic acid for 15 min at 0 °C (ref. 26), or with 50% acetic and 1.25% mercaptoacetic acid for 4 min at 20 °C (ref. 25). After centrifugation, the supernatants were analyzed for iron and radioactivity; the pellets were washed before being assayed.

Surprisingly enough, most of the cyanide is not released in the supernatant even by these very harsh treatments, and a minor but significant amount of iron remains protein bound. A value of 2 ± 0.5 moles cyanide per gatom of iron is found in the protein after both procedures.

The anomalous behaviour of the non-haem iron to acid extraction can be induced in soluble, highly purified succinate dehydrogenase[6], by reaction with cyanide (last line of Table I).

Spectral modifications

Incubation with cyanide affects the spectrum of soluble succinate dehydrogenase. In Fig. 3 difference spectra at various times of incubation with KCN are reported. As it can be seen, cyanide induces a slow decrease of absorbance all over the visible spectrum of this metalloflavoprotein. It has been checked that succinate dehydrogenase, even at a higher level of purity[6], gives the same type of difference spectrum. In the same figure the non-haem iron spectrum of succinate dehydrogenase is shown for comparison. The two spectra, although similar, show some differences, particularly in the 400-nm region. It follows that the effect of cyanide is not merely a partial destruction of the non-haem iron chromophore of the enzyme, but rather a

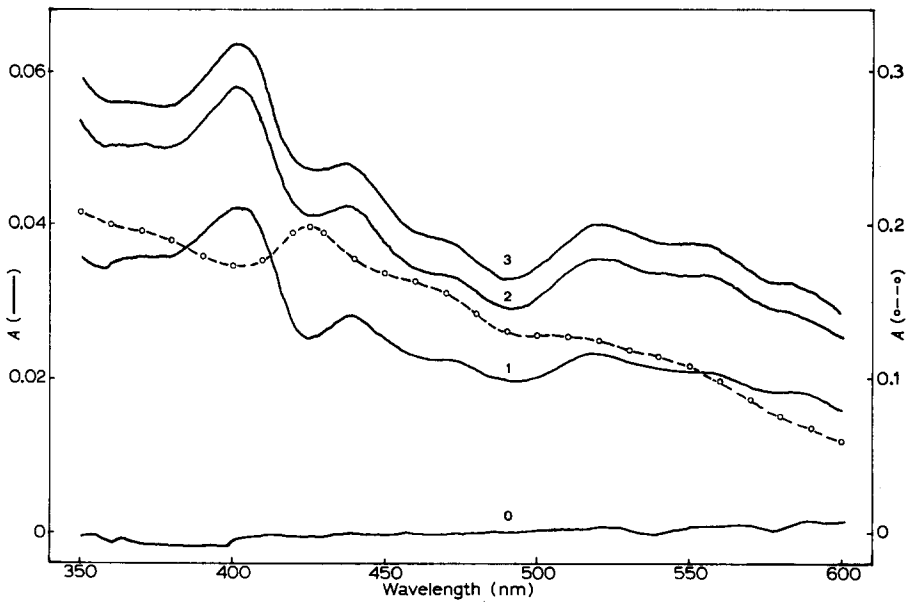

Fig. 3. Difference spectrum of soluble succinate dehydrogenase treated with cyanide. Each cuvette contained 4 mg·ml^{-1} succinate dehydrogenase at the gel eluate stage[6], in 0.1 M phosphate buffer (pH 7.4), at 20 °C in air. To the reference 1 M KCN (pH 7.4) was added to a final concentration of 50 mM. Another aliquot of 1 M KCl was used to dilute the enzyme of the sample cuvette. The difference spectra were recorded on the 0–0.1 scale of a Beckman Acta III. Trace 0, baseline; Trace 1, 7-min incubation; Trace 2, 28-min; Trace 3, 40-min. The spectrum (○– – –○) was obtained by plotting the difference between the spectra recorded before and after addition of *p*-chloromercuribenzoate (final concentration 2 mM) to the KCl- treated enzyme. To prevent development of turbidity upon addition of mercurial, the enzyme solution was made 1.3 M in urea[11].

hypochromic modification of it. An involvement of the flavin chromophore cannot be excluded, however, the major changes occur at wavelengths not characteristic of the FAD spectrum.

Molecular species

The elution pattern of the preparative gel filtration suggested the presence of more than one active component. This observation was confirmed by isoelectrofocusing and electrophoresis on cellulose acetate[6]. It was, therefore, attempted to resolve the various active species and to determine their molecular weights.

Centrifugation of the cyanide-enzyme in a linear glycerol density gradient gives the sedimentation pattern shown in Fig. 4. Two partially resolved peaks are always present in a percent ratio of approximately 65:35 with the predominance of the heavier species.

Double logarithmic plots of molecular weights *versus* migration from meniscus[29] allow the calculation of the unknown molecular weights. These values, out of ten determinations with five different enzyme preparations, averaged (± 10%): 180 000 and 140 000 daltons, respectively.

Analytical gel filtration of cyanide-enzyme gives only one asymmetric peak, which trails on its descending side (Fig. 5, Curve A), suggesting the presence of

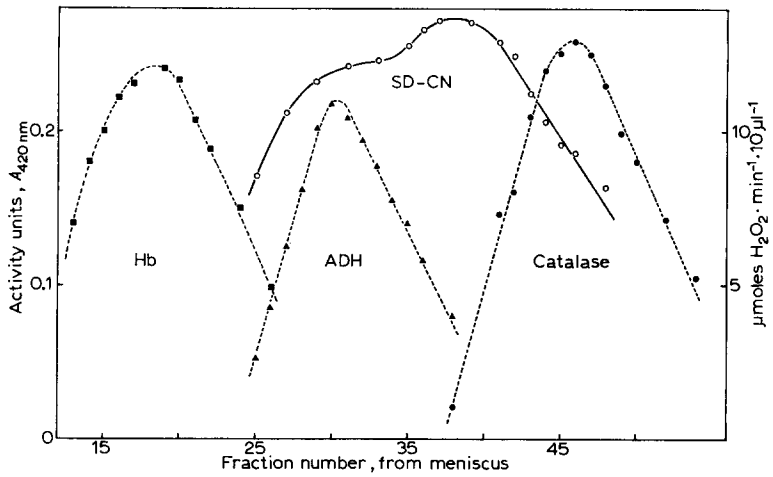

Fig. 4. Sedimentation pattern of cyanide-enzyme in density gradient centrifugation. Cyanide-enzyme ($\Delta A_{600\,nm} \cdot min^{-1} \cdot 30\,\mu l^{-1}$, ○ – – ○); haemoglobin ($A_{420\,nm}$, ■– – –■); alcohol dehydrogenase ($\Delta A_{340\,nm} \cdot min^{-1} \cdot 5\,\mu l^{-1}$, ▲– – –▲); catalase (●– – –●). Density gradient centrifugation was carried out according to Martin and Ames[29]. 0.1 ml of the protein mixture (containing 0.9 mg cyanide-enzyme) was layered on top of the preformed glycerol density gradient (5–25%, v/v) in deaerated 50 mM phosphate (pH 7.4). Centrifugation was run at 1 °C in the 3 × 6 ml swing-out bucket rotor at 45 000 rev./min for 15–16 h.

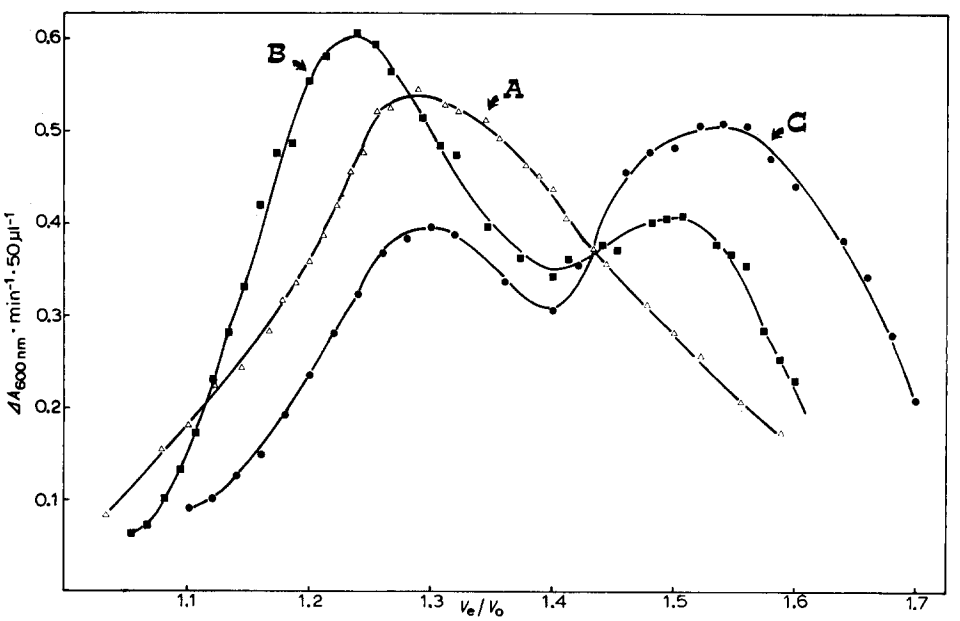

Fig. 5. Elution patterns of cyanide-enzyme (before and after treatment) from Sephadex G-200. Conditions were as in Fig. 6. 9 mg protein were applied in each experiment. Curve A (△—△), untreated cyanide-enzyme; Curve B (■—■), cyanide-enzyme was incubated with 0.4 M $NaClO_4$ in 50 mM sodium phosphate buffer (pH 7.4) under N_2, 30 min at 2 °C. The incubation mixture was then filtered on the G-200 column, equilibrated with the same buffer containing the chaotropic agent. Dextran blue, bovine serum albumin and cytochrome *c* were run in the same conditions as markers (Expts B and C). Curve C (●—●), cyanide-enzyme was incubated with 0.8 M sodium trichloroacetate in 50 mM phosphate buffer (pH 7.4) under N_2, 120 min at 2 °C. The sodium trichloroacetate during elution was 0.4 M.

unresolved lighter species. The molecular weight at the top of the peak corresponds to 178 000 (Fig. 6).

If the cyanide-enzyme is treated with chaotropic agents and gel filtration is performed in their presence, a good separation of two major species is obtained (Fig. 5, Curves B and C). $NaClO_4$ or trichloroacetate, which are both very strong chaotropic agents[32] were used interchangeably, after it had been checked that they did not affect the activity of the cyanide-enzyme even in prolonged incubation. The

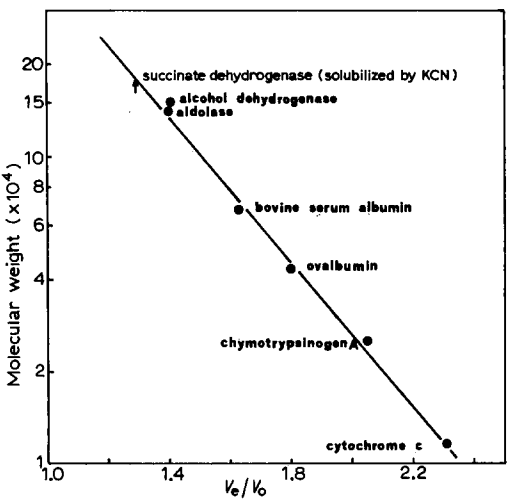

Fig. 6. Molecular weight estimation of cyanide-enzyme by Sephadex G-200 filtration. The column (100 cm × 1.6 cm) was equilibrated in 50 mM phosphate buffer (pH 7.4) at 2 °C in N_2. Each protein (1 ml solution) was applied separately. Fractions of 1 ml were collected. The values of the molecular weight of the protein markers are as reported in refs 30, 31.

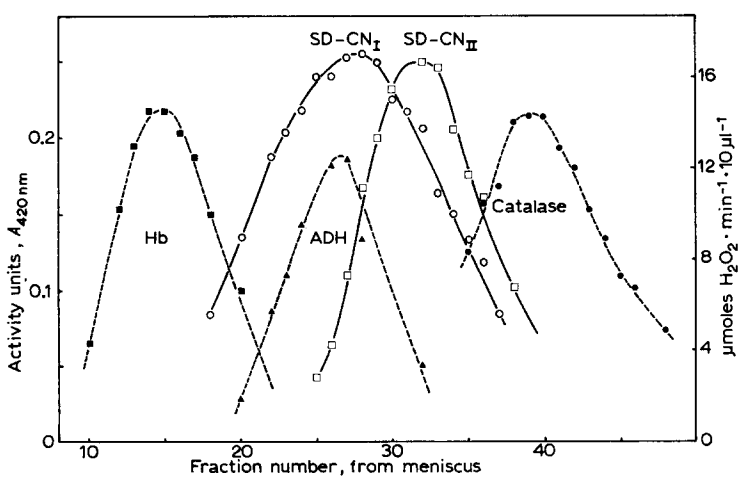

Fig. 7. Sedimentation patterns of cyanide-enzyme after treatment with chaotropic agents. Conditions are as in Fig. 4. The first peak eluted from Sephadex G-200 column (Fig. 5, Curve B) and the second peak (Fig. 5, Curve C) were desalted, concentrated by $(NH_4)_2SO_4$ and ultracentrifuged. Their patterns are reported on the same graph and indicated as $SD\text{-}CN_{II}$ and $SD\text{-}CN_I$, respectively. Hb, haemoglobin; ADH, alcohol dehydrogenase.

ratio between the two species depends on the concentration of the chaotropic agents and a larger amount of the lighter component is obtained at very high concentrations of chaotropic agents by partial conversion from the heavier species. In the conditions of the experiment shown by Curve B in Fig. 5, the ratio is almost equal to the value found in the gradient ultracentrifugation, performed in the absence of the chaotropic agents.

The heavier species resolved by gel filtration in chaotropic agents (Fig. 5, Curve B) was separated and filtered on the analytical Sephadex G-200 column. It yielded a single symmetrical peak of mol. wt 180 000 $\pm$ 10% (Fig. 6).

The other species peaks around a V_e/V_0 of 1.5 (Fig. 5, Curves B and C), which, also with reference to markers run in the same conditions, corresponds to a mol. wt of about 100 000.

The molecular weight of the species isolated by gel filtration in chaotropic agents was determined also by gradient ultracentrifugation as already described. Both forms give a single symmetrical peak. But while the molecular weight of the heavier species was confirmed, the lighter one sediments at an apparent mol. wt of 140 000–150 000 (Fig. 7), reproducing the sedimentation value of the lighter component found in cyanide-enzyme before treatment with chaotropic agents (Fig. 4).

DISCUSSION

Succinate dehydrogenase solubilized by KCN is modified with respect to the following properties: it has a lower catalytic centre activity, a labile sulphide/non-haem iron ratio lower than unity and contains strongly bound cyanide.

The excess of iron over acid-labile sulphide indicates a peculiar modification of the iron–sulphur group of the protein. Hough and Rabinowitz[33] have shown that in clostridial ferredoxin the iron–sulphur prosthetic group is destroyed and reconstituted in an all-or-none mode, and no iron-poor or sulphide-poor protein molecules are obtained. On the other hand, Petering *et al.*[34] found that in extensively oxygen-denatured iron–sulphur proteins, labile sulphide is converted to an oxidized species, called sulphur-zero, without iron being removed. This form of sulphur has not been tested for in cyanide-enzyme; evidence that will be discussed later suggests that cyanide replaces part of the labile sulphide as ligand for non-haem iron.

Cyanide-enzyme is polydisperse. Two major active components of apparent mol. wt 180 000 and 140 000 were found by ultracentrifugation in the density gradient. The heavier species contributes to approximately 65% of total activity.

Gel filtration in chaotropic agents, which weaken hydrophobic interactions[32], resolves two components of mol. wt 180 000 and 100 000, respectively. This latter species behaves as the purified flavoprotein isolated in the absence of cyanide, with respect to the following properties: the molecular weight is similar to the minimum molecular weight of the pure flavoprotein (about 97 000) [11,35] and both sediment in a density gradient with an apparent mol. wt of 140 000 (ref. 7). This behaviour is explained by assuming that the lighter species of cyanide-enzyme undergoes the same dynamic monomer–dimer equilibrium proposed for the purified succinate dehydrogenase[36]. Chaotropic agents appear to shift this equilibrium toward the monomer, suggesting a prevailing involvement of hydrophobic bonds in the dimer formation.

The heavier component of cyanide-enzyme can be isolated and does not yield

the lighter species spontaneously. Only high concentrations of chaotropic agents bring about a partial conversion of this form to the monomer. The latter apparently does not reassociate to form the original mol. wt 180 000 species. Thus the two active components of cyanide-enzyme are not simply interconvertible.

Two alternative hypotheses can be formulated about the nature of the heavier species of cyanide-enzyme. Originally, we suggested that this form resulted from the association of succinate dehydrogenase with some other membrane component[7]. However, the more likely interpretation of all the data discussed above, is to identify the heavier species with a stabilized dimer of the flavoprotein. The stabilization should arise during solubilization of the enzyme by KCN from the membrane, and may be induced by the incorporation of cyanide in succinate dehydrogenase.

It remains to establish the possibility of the pre-existence in the membrane of the dimer and its relevance to the mechanism of action of succinate dehydrogenase.

With regard to the chemical species involved in the interaction with succinate dehydrogenase, different workers have proposed either HCN[1,5] or cyanide anion[2]. It seemed essential to resolve this discrepancy for a better understanding of the mechanism of solubilization. Our results point out that the solubilization of succinate dehydrogenase is a function of CN^- concentration and no influence of pH is observed, at least in the range from 6 to 8.

The action of CN^- cannot be compared to any of the chemical agents, which are used to resolve succinate dehydrogenase from the membrane: organic solvents[37,38], hydroxyl ions[39] and chaotropic ions[11]. CN^-, to our knowledge, is not considered a chaotropic agent[40]; anyhow, we can exclude a similarity of action on the basis of the following observations. The optimal concentration of this ion is several hundred times lower than that of the more efficient chaotropic agents used by Davis and Hatefi[11]. Furthermore, succinate prevents only the solubilization by KCN and attempts to resolve the flavoprotein from the same material (Keilin–Hartree heart muscle preparations) with sodium perchlorate at high concentrations, gave poor results.

The process of solubilization of succinate dehydrogenase follows pseudo-first-order kinetics and the order of reaction with respect to CN^- is one. Thus apparently, the interaction of just one CN^- with the flavoprotein is sufficient to bring about resolution from the membrane. The discrepancy between this value and the amount of cyanide actually found in the cyanide-enzyme is only apparent. Additional reaction of CN^- with the solubilized enzyme has to occur. Indeed it has been reported[27,41] that succinate dehydrogenase, solubilized by butanol, is able to incorporate several moles of cyanide.

The locus of action of cyanide on succinate dehydrogenase has been only conjectural[2,3]. All our data lead to the conclusion that the iron–sulphur complex is certainly involved in the interaction with cyanide. The lower content in labile sulphide of cyanide-enzyme *per se*, is not direct evidence of cyanide action since other soluble succinate dehydrogenase preparations are modified in this respect[42]. Rather, other features can be specifically ascribed to cyanide. The lower ratio SH_2:Fe has already been discussed above. Moreover, KCN induces a hypochromic modification of the spectrum of soluble succinate dehydrogenase, which suggests a perturbation of the non-haem iron chromophore. The difference spectrum seems not to arise merely from a destruction of the group and is not comparable to spectral changes induced by competitive inhibitors[43]. Non-haem iron of cyanide-enzyme and of succinate dehydro-

genase treated with KCN, shows an anomalous behaviour. Indeed part of the iron acquires stability to mild acid extraction. An interpretation of this phenomenon was possible when it was found that the incorporated cyanide was also stable to the same treatments and was recovered bound to protein in a ratio with iron of 2:1. As it should be recalled, there is some evidence that the amount of cyanide bound in the cyanide-enzyme is about twice the difference between non-haem iron and labile sulphide. Therefore, it can be suggested that CN^- (monofunctional) substitutes labile sulphide (bifunctional) as ligand for non-haem iron, and likely stabilizes iron to acid extraction. Finally the resolution of succinate dehydrogenase from the membrane is inhibited by the iron chelator 2-thenoyltrifluoroacetone[5].

As an alternative explanation, cyanolysis of a disulphide by nucleophilic displacement has been proposed[3]. A number of observations stand against this hypothesis. The procedure of KCN extraction[6] is relatively mild if compared to the treatments used in model reactions[44,45] and in the latter cases, the radioactivity (^{14}C) is released from protein. Other nucleophiles such as sulphite and mercaptoethanol do not mimic the cyanide reaction. Recent reports in the literature establish that a cyanolysis of disulphide bridges can actually occur even in conditions similar to ours. However, these kinds of disulphide bonds are very reactive as in the monothionitrobenzoate derivative of isocitrate dehydrogenase[46] or in the postulated persulphide group of xanthine oxidase[47]. On the other hand, a metal has been always claimed to be the site of action of cyanide in other metalloflavoproteins[48,49].

In conclusion, the action of CN^- on the relationships between succinate dehydrogenase and the membrane seems to be an indirect one: binding to iron should induce a conformational change in the protein, thus labilizing its linkages with the other membrane components.

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