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Evidence for the Presence of Di- and Triphospho Pyridine Nucleotide Dehydrogenase Derivatives as Consistent Contaminants of Purified Beef Heart Cytochrome-*c* Oxidase

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Abstract

Purified beef heart cytochrome-*c* oxidase preparations derived by three different laboratories contain NADH–$K_3Fe(CN)_6$, NADH–nitrobluetetrazolium, and NADPH–nitrobluetetrazolium reductases. This is true of preparations exhibiting heme aa_3 to protein ratios considered indicative of an excellent purity. An apparent association of cytochrome-*c* oxidase and one or more of the contaminants persists through immunodiffusion and nondenaturing electrophoresis and, in addition, in one instance copurification of NADH–$K_3Fe(CN)_6$ reductase and cytochrome-*c* oxidase to a constant ratio of specific activities was demonstrated. Cytochrome-*c* oxidase can be freed of the contaminants by equilibration with an NAD^+-affinity matrix. As a concomitant of equilibration with

the matrix, the K_M of cytochrome-*c* oxidase for ferrocytochrome-*c* is invariably decreased. Rate constants at low ferrocytochrome-*c* concentrations are consistently enhanced in all oxidase preparations upon equilibration with the NAD^+ matrix. However, the effects of such equilibrations on the extrapolated V_{max} varies from one preparation to another. Polyacrylamide gel electrophoresis in SDS–urea systems establishes that each of the preparations contains a minimum of three contaminants, each of an apparent formula weight of greater than 40,000 Daltons. NADH–NBT reductase was found to have a formula weight of approximately 46,000 Daltons. Their properties establish that NADH–$K_3Fe(CN)_6$ and NADH–NBT reductases are separate proteins; the separate identity of NADPH–NBT reductase has not yet been determined.

Introduction

We have reported the ability of rabbit antisera to beef heart cytochrome-*c* oxidase (E.C. 1.9.3.1) to cross-react quantitatively with rat liver cytochrome-*c* oxidase [1]. This cross reactivity was demonstrated in exploring the potential utility of such antisera in intended studies of synthesis of rat liver cytochrome-*c* oxidase. However, in determining the character of the cross reaction, it became apparent that the antisera were not monospecific. Since we were uncertain of the extent and character of the contamination of the beef heart cytochrome-*c* oxidase preparations, as well as of the antisera derived by their use, the problem had to be clarified prior to any studies of synthesis. The multiple specificity of the antisera was first made manifest when superfluous precipitin lines were observed on immunodiffusion plates when either crude or purified oxidase preparations from either rat liver or beef heart were diffused against antisera to beef heart cytochrome-*c* oxidase.

The first clue to a possible cause of the problem appeared when it was found that a contaminant of rat liver cytochrome-*c* oxidase preparations made with Triton detergents (at least in our hands) was a protein or proteins which could oxidize NADH in the presence of suitable electron acceptors. The relevance of this observation to the problem became apparent when formazan precipitation was observed atop precipitin lines when immunodiffusion plates were covered with an aqueous solution of NADH and nitrobluetetrazolium (NBT). We have now accrued abundant evidence that highly purified beef heart cytochrome-*c* oxidase obtained by several conventional purification procedures will invariably contain one or more enzymic entities capable of oxidizing NADH and NADPH with either $K_3Fe(CN)_6$ and/or NBT as electron acceptors. These results should be of prime importance in assessing the validity of studies wherein a homogeneous cytochrome-*c* oxidase is required either as such or as an antigen for the derivation of a truly monspecific antiserum. Preliminary reports of this work have appeared in the literature [2, 3].

Materials and Methods

Materials

Cytochrome-*c* (type III), NAD^+, NADH, NADPH, urea, and nitrobluetetrazolium were obtained from Sigma Chemical Co. Agarose and Dowex AG501 were obtained from Bio-Rad Laboratories. Two affinity matrices were obtained from P-L Biochemicals, Inc. (AGNAD, type I, and AGATP); each contained 7 μmole of functional group/ml of wet gel. Various other chemicals and reagents and their sources were as follows: Emasols 4130 and 1130, Kao-Atlas Co., Ltd., Tokyo; Coomassie Blue (R-250), Colab Laboratories; SDS, Fisher Scientific Co.; acrylamide and *N,N'*-methylenebisacrylamide, Aldrich Chemical Co.; *N,N,N',N'*-tetramethylethylenediamine and β-mercaptoethanol, Eastman Chemical Co.; Ponceau S, Gallard-Schlesinger Chemical Manufacturing Co.; Triton-X-100, M and H Distributors, Inc., Winston-Salem, N.C.; Tween 80, Baker Chemical Co.; MES [2-(*N*-morpholino)ethanesulfonic acid], Calbiochem; Duolite S-10, Diamond Alkali Co.

*Cytochrome-*c *oxidase Preparations and Assay*

Throughout this experimentation cytochrome-*c* oxidase preparations have been suspended for use in 100 mM phosphate, pH 6.5 : 0.05% Tween 80 (phosphate-Tween). Initially, experiments were performed with that portion of the cytochrome-*c* oxidase preparations which is soluble to 60 min centrifugation at 105000 × *g*. The preparations were solubilized by 60 sec sonication (for 10 sec intervals, with intervening cooling periods) at ice temperature in 80–100 volumes of phosphate–Tween. Experiments so performed are identified in the text; most experiments were performed with direct dilutions of preparations made in phosphate–Tween.

Cytochrome-*c* oxidase activity was assayed according to Yonetani [4] in a 1 ml volume containing 50 mM phosphate buffer, pH 6.0 and 0.5 mM EDTA. Ferrocytochrome-*c* was prepared either by use of Duolite S-10 as described by Chantrenne [5] or according to Yonetani [6]. Spectra of cytochrome-*c* oxidase and measurements of its activity were obtained at room temperature by use of a Cary model 14 spectrophotometer employing cuvettes with a 1-cm light path. Cytochrome-*c* oxidase concentrations (expressed as heme aa_3) were determined [7] either from difference spectra (ϵ_{mM} of 83.5 at 443 nm or 12 at 605 nm) or reduced spectra (ϵ_{mM} of 100 at 443 nm or 16.5 at 605 nm).

Other Enzyme Assays

Assays for each reductase were performed at 25° C by use of a Beckman 25 spectrophotometer (1-cm cells); all results have been corrected for any nonenzymic activity. NADH–$K_3Fe(CN)_6$ reductase activity was assayed [8] by following the decrease in absorbance at 420 nm upon addition of enzyme to a system of 1 ml final volume containing 61 mM phosphate buffer, pH 7.3; 0.3 mM NADH; 1.75 mM $K_3Fe(CN)_6$. NADH–NBT and NADPH–NBT reductase activities were assayed by incubation of enzyme in 0.5 ml volume containing either 0.3 mM NADH or 0.15 mM NADPH; 200 μM MES, pH 6.4; 1% Emasol 4130; and 0.025% NBT. Other specific conditions or deviations from the foregoing are indicated with the results. Incubations were started by addition of substrate, and formazan production was measured by following the increase in absorbance at 510 nm. In contrast to the foregoing, formazan production in initial experiments was measured by its extraction by ethyl acetate from the acidified reaction medium. Results which were so obtained are clearly identified in the text. In either case, assay results are expressed as units of increase in absorbance at 510 nm/minute/mg protein. Extensive exploratory experimentation to delineate the optimal assay system established that the indicated concentrations of NADH, NADPH, and NBT were saturating and that the final assay conditions yielded zero-order kinetics.

Immunochemical Procedures

The preparation and characterization of rabbit antisera to beef heart cytochrome-*c* oxidase was performed as detailed previously [9]. Immunodiffusion plates contained the following: 0.7% agarose; 0.05 M phosphate, pH 7.4; 0.001% merthiolate. Cytochrome-*c* oxidase was applied to the wells of plates in 2% Tween 80 to facilitate diffusion.

Electrophoresis

Nondenaturing polyacrylamide gel electrophoresis (PAGE) was performed according to Zamudio and Williams [10]. Denaturing PAGE in the presence of 0.1% SDS–8 M urea was performed as detailed by Swank and Munkres [11]. Urea, deionized by treatment with Dowex AG501, was used in the buffer mixture employed for sample preparation. Acrylamide and SDS were recrystallized for use from chloroform and absolute ethanol, respectively. For the purpose of electrophoresis (50–100) μg of lyophilized protein was dissolved in 0.05 ml of 0.01 M H_3PO_4 : 1% (w/v) recrystallized SDS : 1% (v/v) 2-mercaptoethanol : 8 M urea; the mixture was adjusted to pH 6.8 with solid Tris for use. Dissociation was accomplished by heating at 70° C for 15 min;

one drop of 50% glycerol and 3 μl bromphenol blue were then added to the sample prior to its placement on the gel. Electrophoresis was carried out for 8 hr at 2 mA/gel.

Staining Procedures

Separate but identical immunodiffusion plates were stained for cytochrome-*c* oxidase, NADH–NBT reductase, and protein. A stain for cytochrome-*c* oxidase employing *N,N*-dimethyl-*p*-phenylene-diamine and α-naphthol (Nadi reaction) was performed as described [9]. NADH–NBT reductase was visualized by overlaying a plate with a solution containing 0.8% agarose, into which (while the agarose was at a temperature of 40–50° C) had been mixed sufficient NADH and NBT to give final concentrations of 0.25% and 0.5%, respectively. When appropriate, polyacrylamide gels were sectioned longitudinally and stained for cytochrome-*c* oxidase and NADH–NBT reductase as described above. SDS–urea gels were fixed and stained for protein by use of Coomassie Blue according to Swank and Munkres [11].

Other Procedures

Protein concentrations were determined by either the biuret [12] procedure or a modified Lowry procedure [13]. Samples containing substantial amounts of cytochrome-*c* oxidase were pretreated with H_2O_2.

Equilibrations of cytochrome-*c* oxidase preparations with AGNAD or AGATP were accomplished in phosphate–Tween. The matrix was well washed with distilled water and phosphate–Tween prior to use. Equilibrations were accomplished by circulating the oxidase preparation through a column bed of matrix supported in a disposable graduated pipet. Medium at a constant flow of 1.5 ml/min, at 5° C and ambient pressure, was cycled through the matrix by use of a peristaltic pump.

Results

Table I presents the sources and methods of preparation of each of the cytochrome-*c* oxidase preparations used as well as their contents of heme aa_3 per mg of protein. While several preparations might not be considered of the highest caliber, preparations 3–7 approach the values of 10–11 nmole heme aa_3/mg protein which are generally accepted as characteristic of an excellent oxidase. Emphasis is placed on the content

TABLE I. The character of beef heart cytochrome oxidase preparations utilized in this study

Number	Method of preparation	Ref.	nmole heme a per mg protein
1	Triton detergents	14	7.0
2	Yonetani	4	7.7
3, 4	Fowler et al. (modified)	15, 16	9.7, 9.9
5	Yonetani (modified)	4, 17	10.9
6, 7	Fowler et al. (modified)	15, 18	10.3, 11.3

of heme aa_3 as a measure of quality for two reasons. First, although the preparations were always shipped in dry ice and stored at $-70°C$ (preparations 5, 6, and 7 are exceptions, they were stored under liquid N_2), each had a separate history of ageing, etc., and, consequently, variations in phospholipid content and activity were to be expected. Second, the quantities of several preparations were limited and thus extrapolated maximal velocities or specific activities could not be determined for them.

TABLE II. Specific activities of contaminant reductases in the cytochrome-*c* oxidase preparations of Table I[a]

	Reductase activities			
Preparation	Substrate: Acceptor:	NADH $K_3FE(CN)_6$	NADH NBT	NADPH NBT
1		5.4[b]	8.8[c]	0.4[c]
2		1.0	0.2	0.2
3		0.1	1.1	0.3
4		0	0.2	0.02
5		2.7	0.1	0.02
6		3.6	0.3	0.08
7		4.0	0.6	0.5

[a] Conditions: All assays were conducted as indicated in text. Analyses of preparations 1–4 were performed with that portion of the sonicated preparation which was unsedimented upon centrifugation at 105,000 x *g* x 60 min.

[b] μmole $K_3Fe(CN)_6$ reduced/min/mg protein.

[c] Units $\Delta A_{510\,nm}$/min/mg protein. Determined for preparations 1–4 by means of the ethyl acetate extraction procedure.

The Specific Activities of the Reductases of Various Cytochrome-c Oxidase Preparations

To date, every cytochrome-*c* oxidase preparation we have tested can oxidize both NADH and NADPH with NBT as an electron acceptor. Table II presents the activities of NADH–$K_3Fe(CN)_6$ reductase, NADH–NBT reductase, and NADPH–NBT reductase in each of the preparations. It can be seen that each, with the exception of preparation 4, contained demonstrable levels of each of the enzymic activities, and, although there were variations in specific activities from preparation to preparation, even that with the highest heme aa_3 to protein ratio contained demonstrable levels of each contaminant.

Evidence for the Association of Cytochrome-c Oxidase and its Contaminants

The association of cytochrome-*c* oxidase and NBT reduction is easily demonstrated immunochemically. Cytochrome-*c* oxidase preparations will characteristically yield two precipitin lines upon diffusion against antiserum, reflecting the propensity of the aged enzyme to aggregate. When separate, identical plates are stained for protein and the Nadi reaction, both precipitin lines are postive in their reaction to both stains. The point of importance, however, is that at least one or, more commonly, both such precipitin lines will also develop color when a third such plate is stained with NADH and NBT [19]. Comparisons of many sets of such plates have established that a positive reaction to the NADH–NBT stain is always coincident with the positive reactions to the protein and Nadi stains and similar results have been obtained on the few occasions wherein NADPH has been substituted for NADH. Association will also persist through procedures of nondenaturing electrophoresis. Cytochrome-*c* oxidase, when subjected to polyacrylamide gel electrophoresis according to Zamudio and Williams [10], exhibits two broad Nadi-positive bands. When such gels are sectioned, however, both bands are found positive to the NADH–NBT stain as well.

A specific example of the associative tendency of the NADH–$K_3Fe(CN)_6$ reductase and cytochrome-*c* oxidase is shown in Table III, wherein the activities of cytochrome-*c* oxidase and of the various reductases at successive steps in the derivation of preparation 2 are presented. The relationship between the specific activity of cytochrome-*c* oxidase to that of the NADH–$K_3Fe(CN)_6$ reductase for each step of the isolation procedure is presented in the column on the right. It can be seen that in fractions S_1–S_3 this ratio increased as the fractionation proceeded, but at steps S_4, S_5, and S_6 the ratio became a constant. Similar calculations for the NADH– and NADPH–NBT reductases exhibited a decline with increasing purification, indicating

TABLE III. Copurification of NADH–$K_3Fe(CN)_6$ reductase and cytochrome-*c* oxidase[a]

Fraction	Cytochrome-*c* oxidase[b]	Substrate: Acceptor	NADH		NADPH	Ratio
			$K_3Fe(CN)_6$[c]	NBT[d]	NBT[d]	
S_1	2.8		7.0	1.4	2.3	0.4
S_2	3.5		10.3	1.3	2.1	0.3
S_3	3.8		0.8	0.8	0.8	4.9
S_4	6.3		0.9	0.4	1.2	7.0
S_5	6.3		0.9	0.4	1.6	7.0
S_6	7.2		1.0	0.2	0.8	7.2

[a] Conditions: S_1, initial particle preparation; S_2, 50% ammonium sulfate precipitate; S_3, 35% ammonium sulfate precipitate; S_4 and S_5, precipitates obtained by refractionation with 26% and 33% ammonium sulfate; S_6, preparation 2, the dialysate derived from S_5. NADH–NBT reductase assay: 50 mM MES, pH 6.4; 0.5 ml reaction volume; incubated 10 min at 22°C. NADPH–NBT reductase assay: 50 mM Tris, pH 7.0; 2 ml reaction volume; incubated 10 min at 30°C. Formazan production was determined by extraction with ethyl acetate. Cytochrome-*c* oxidase was assayed at a concentration 136 μM ferrocytochrome-*c*. Specific activity ratio: cytochrome-*c* oxidase to NADH–$K_3Fe(CN)_6$ reductase.

[b] μmoles ferrocytochrome-*c* oxidized/min/mg protein.

[c] μmoles $K_3Fe(CN)_6$ reduced/min/mg/protein.

[d] ΔA_{510nm} /min/mg protein.

their apparent continued removal in the later steps of purification. It should be noted that, while we have used the term association to describe some of our observations, we do not imply that we consider our own findings to be proof of a capacity for definite protein–protein interactions between cytochrome-*c* oxidase and its contaminants. Our findings may stem equally well from very close similarities in the physical properties of the proteins.

Treatment of Cytochrome-c Oxidase Preparations with Affinity Matrices

In view of the character of the contaminants found in cytochrome-*c* oxidase preparations, we have explored the potential utility of affinity matrices to purify cytochrome-*c* oxidase. Several agarose matrices containing NAD^+ have been tested extensively, and one containing ATP has been investigated to a limited extent. We find that an NAD^+ matrix (AGNAD) is well suited to the purpose at hand; preliminary experimentation indicates that the ATP matrix (AGATP) may serve equally well. Figure 1 illustrates the extent of binding of various components when preparation 7 was equilibrated with AGNAD (1A) and with AGATP (1B). AGNAD can be seen to have reduced the specific activities of the various reductases from 92% to 100%. On the other hand, AGATP was apparently somewhat less efficient in the binding of the NBT reductases. However, it should be noted that the AGATP was being

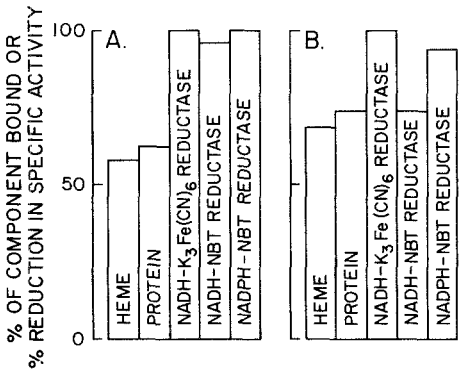

Figure 1. The Binding of various components of cytochrome-*c* oxidase of preparation 7 by AGNAD and AGATP. Conditions: 1A—14.8 nmole heme aa_3 were equilibrated with 1.5 ml AGNAD for 30 min in a volume of 6.5 ml; 1B—102 nmoles heme aa_3 were equilibrated with 4.5 ml AGATP for 30 min in a volume of 10 ml.

utilized under a heavier load of heme aa_3 (and protein) per unit of matrix than the AGNAD. At comparable ratios of heme aa_3 to matrix volume, AGATP is as efficient as AGNAD in binding of the reductases. In addition to the foregoing, the results of Fig. 1 establish that both matrices bind substantial quantities of both protein and heme aa_3—a point we will return to subsequently.

In exploring the ability of the matrices to free cytochrome-*c* oxidase of the various reductases, we observed that the velocity of ferrocytochrome-*c* oxidation was often enhanced following equilibration with an affinity matrix.. As we have reported elsewhere, the enhancement of activity at single substrate concentrations was frequently quite pronounced [20]. We have now determined the effects of AGNAD on the kinetic constants of cytochrome-*c* oxidase in greater detail. The results of equilibration of two preparations, 5 and 7, with AGNAD are depicted in Fig. 2. These plots are each representative of at least two or more such experiments. With each preparation, it can be seen that treatment with AGNAD enhanced substantially the activity of cytochrome-*c* oxidase at low substrate concentrations but the ultimate influence on the extrapolated maximal velocity differed substantially with the two preparations. At the same time, regardless of the character of the effect of AGNAD treatment on the V_{max}, the equilibration invariably exerted a favourable influence on the K_M of cytochrome-*c* oxidase for ferrocytochrome-*c*. These points can be seen more clearly in an inspection of Table IV wherein the constants derived from the plots of Fig. 2 are presented. The difference in the effects of AGNAD on the respective maximal velocities of preparations 5 and 7 is quite apparent. That of preparation 5 was increased 80% while the V_{max} of preparation 7 was

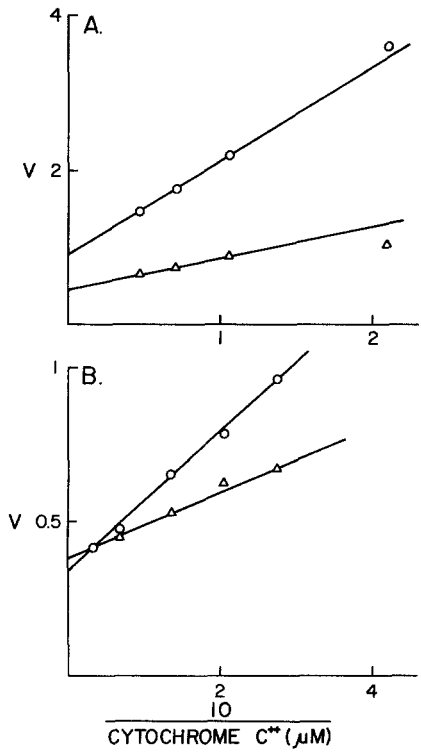

Figure 2. Reciprocal plots of cytochrome-*c* oxidase activity before and after equilibration with AGNAD. Conditions: 2A—9.1 nmole heme aa_3 of preparation 5 were equilibrated with 0.5 ml of AGNAD for 105 min in a volume of 13 ml; 2B—14.8 nmole heme aa_3 of preparation 7 were equilibrated with 1.5 ml AGNAD for 30 min in a volume of 6.5 ml. Control, ○—○; equilibrated △—△. *V*, nmoles cytochrome-c^{++} oxidized/sec/nmole heme aa_3.

TABLE IV. The effects of AGNAD on the kinetic constants of cytochrome-*c* oxidase activity (values obtained from the reciprocal plots Fig. 2)

Preparation	Matrix	V_{max}[a]	K_M (μM)
5 (Fig. 2A)	Control	116	15.1
	AGNAD	213	8.3
7 (Fig. 2B)	Control	294	6.4
	AGNAD	241	2.6

[a] nmoles ferrocytochrome-*c* oxidized/sec/nmole aa_3.

diminished 19%. However, with both preparations, equilibration with AGNAD wrought a 50% decrease in the K_M for substrate. Exploratory equilibrations of preparation 7 with AGATP establish that that process has effects on K_M and V_{max} which are essentially identical to those resulting from equilibration with AGNAD.

The removal of the several contaminant reductases by AGNAD from cytochrome-*c* oxidase can be correlated with the removal of several specific proteins from the oxidase suspension. We have reported recently that mammalian cytochrome-*c* oxidase, when examined by a system of PAGE in 0.1% SDS–8 M urea, contains a total of eight component polypeptides of less than 40,000 Daltons [21]. Examinations of preparations 3–7 by this procedure have shown that these eight components exhibit a remarkable constancy in their relative proportions to each other. Consequently we believe those eight components to be true subunits of mammalian cytochrome-*c* oxidase when it has been brought to the state of purity represented by 10–11 nmole heme aa_3/mg protein. Such experiments have also shown that preparations 3–7 each contain variable amounts of from three to five additional minor components of apparent formula weights in excess of 40,000. It is these latter components of the cytochrome-*c* oxidase preparations which are removed from the system upon equilibration with AGNAD. This has been found to be the circumstance with preparations 5–7; a representative experiment is presented in Fig. 3. Equilibration with AGNAD can be seen to have specifically and quantitatively removed the larger components—results which corroborate and extend the quantitative data of Fig. 1A. Despite the fact that a substantial amount of cytochrome-*c* oxidase was bound by the AGNAD in the experiment of Fig. 1A, a comparison of the two traces of Fig. 3 establishes that this binding produced no detectable changes in the relative proportions of the eight subunits of the oxidase.

Under certain circumstances the loss of from 50% to 60% of the cytochrome-*c* oxidase by non-specific binding to a matrix such as AGNAD or AGATP (e.g., as in Fig. 1) might be acceptable so as to obtain the remaining 40% of the oxidase free of contaminant reductases. Two findings that portend means of improving this situation have been made and are elaborated below in Fig. 4 and Table V. By equilibration of a bed of AGNAD with sufficient cytochrome-*c* oxidase (Fig. 4), it canbe saturated insofar as the binding of further oxidase is concerned (intervals 1–3). Then, upon further additions of the preparation (intervals 4 and 5), 98% of the added oxidase remains unbound while the matrix continues to bind approximately 75% of the added NADH–$K_3Fe(CN)_6$ and NBT reductases. If it is granted that the binding of the various reductases and of cytochrome-*c* oxidase in the experiment of Fig. 4 can be averaged for the total load of the oxidase preparation applied to the matrix bed, then, under such conditions, the matrix reduced the specific

TABLE V. Elution of bound reductases from AGNAD or AGATP[a]

Experiment	Treatment	Elutant	Substrate:	Reductase activities NADH[b]	NADH[c]	NADPH[c]
			Acceptor:	$K_3Fe(CN)_6$	NBT	NBT
1	–	–		3.9	2.6	0.3
	AGNAD	25 mM NAD^+, pH 2.5		3.5	12.8	0.2
2	–	–		3.9	1.7	0.6
	AGNAD	25 mM NAD^+, pH 2.5		2.0	18.6	0.6
3	–	–		4.9	1.0	0.6
	AGNAD	10 mM NADH, pH 8.5		20.4	0.9	0
		10 mM NADPH, pH 8.5		20.9	0.5	0
		25 mM NAD^+, pH 2.5		32.2	30.6	0.6
4	–	–		4.1	2.1	0.3
	AGATP	25 mM NAD^+, pH 2.5		10.8	47.5	1.9

[a] Conditions: Experiment 1—a total of 29 nmole of heime aa_3 was equilibrated for 30 min with 5 ml of AGNAD in a volume of 8.5 ml. Experiment 2—a total of 89 nmole heme aa_3 (added in size equal portions at 30-min intervals) was equilibrated with 1.5 ml of AGNAD in a volume of 6.5 ml. Experiment 3—a total of 23 nmole heme aa_3 (added in five equal portions at 30-min intervals) was equilibrated with 0.5 ml of AGNAD in a volume of 6 ml. Experiment 4—a total of 125 nmole heme aa_3 (added in aliquots of 100 and 25 nmole at 0 and 30 min, respectively) were equilibrated for 60 min with 4.5 ml of AGATP in a volume of 10 ml. Prior to use of the elutants each matrix bed was washed with an appropriate volume of 100 mM phosphate, pH 6.5 : 0.05% Tween 80 and each eluate was diluted upon emergence from the matrix bed with an equal volume of 100 mM phosphate, pH 6.5 : 0.05% Tween 80. Preparation 7 was employed in all experiments.

[b] μmoles K_3 Fe $(CN)_6$ reduced/min/mg protein.

[c] Units ΔA_{510nm}/min/mg protein.

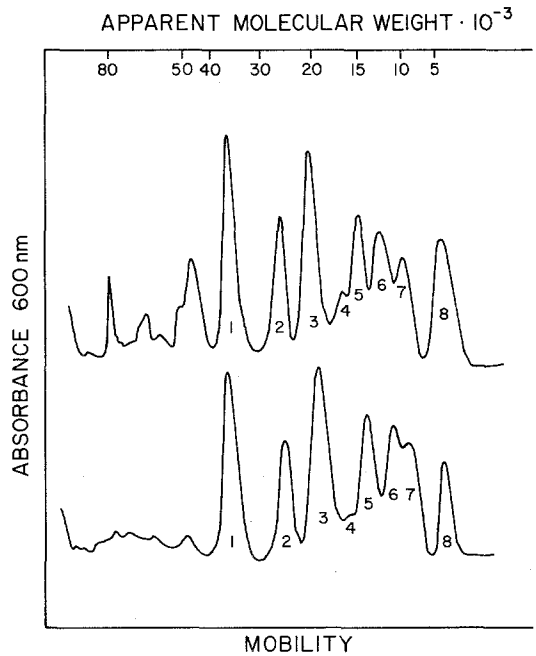

Figure 3. Determination of the specific component polypeptides of cytochrome-*c* oxidase bound by AGNAD. Conditions: Samples of the stock oxidase (upper trace) and of the material not bound by AGNAD in the equilibration experiment of Fig. 1 A (lower trace) containing 50 μg of protein were subjected to electrophoresis in SDS–urea in 8% polyacrylamide gels. Gels were calibrated by use of catalase, D-amino acid oxidase, sperm whale myoglobin, lysozyme (Sigma Chemical Co.), horse liver alcohol dehydrogenase (Worthington Biochemicals), and Cortrosyn (Organon). Densitometry was performed with a Gilford model 2000 spectrophotometer. Control experiments comparing dissociation of cytochrome-*c* oxidase at 70 and 37° C establish that no artifacts of aggregation occur under the conditions employed.

activities of the reductases approximately 80–85%, while at the same time it bound just 10% of the total applied heme aa_3. A second means for a potential improvement in AGNAD's utility lies in our finding that the NADH–$K_3Fe(CN)_6$ and NADH–NBT reductases can be recovered from either AGNAD or AGATP in substantially enriched states. Experiments 1 and 2 of Table V establish that NAD^+ can specifically strip the NADH–NBT reductase from AGNAD. To accomplish this the NAD^+ must be in the unneutralized state; it is inactive at equimolar concentrations at pH values from 7.0 to 3.5. In contrast, NADH or NADPH (pH 8.5) can release the NADH–$K_3Fe(CN)$ reductase essentially free of NADH–NBT reductase (experiment 3). In view of the

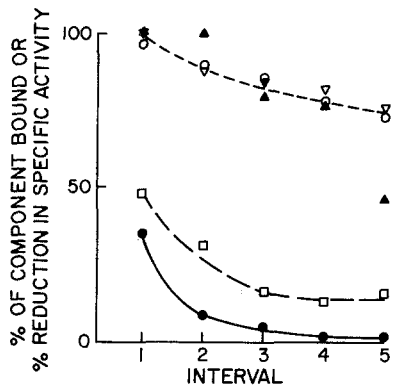

Figure 4. Titration of the binding of cytochrome-*c* oxidase by AGNAD. Conditions: Aliquots of preparation 7 (4.6 nmole heme aa_3) were added at zero time and at each of five subsequent 30-min intervals. Equilibration was accomplished by cyclic flow through a bed of 0.5 ml AGNAD in a volume of 6 ml. Aliquots were also removed at each 30-min interval for analysis, following which an aliquot of enzyme and additional phosphate-Tween were added to restore the 6-ml volume, and cyclic flow was resumed. All calculations involve compensations for the losses of various components removed for analysis at the intermediate time intervals. Symbols: ○—○, NADH–$K_3Fe(CN)_6$ reductase; △—△, NADH–NBT reductase; ▽—▽, NADPH-NBT reductase; □—□, protein; ·—·, heme aa_3.

specificity of NAD^+'s elution capabilities, the release of additional NADH–$K_3Fe(CN)_6$ reductase by NAD^+ in experiment 3 is attributed to an incomplete equilibration of the matrix with NADH and NADPH in the preceding steps. In testing the potential utility of AGATP (experiment 4), the elution of reductases therefrom by NAD^+ was not found to be selective as with AGNAD; a similar situation obtains when ATP is used as an elutant.

The Properties of the Contaminant Reductases in Cytochrome-c Oxidase Preparations

The effects of varying concentrations of NADH and $K_3Fe(CN)_6$ on NADH–$K_3Fe(CN)_6$ reductase activity have been determined for several preparations of Table I. Reciprocal plots wherein activity has been measured as a function of NADH concentration invariably yield aberrant plots with shapes indicative of substrate inhibition. The character of the inhibition has not been studied in detail because of limitations imposed by the available amounts of the preparations. The relationship of specific activity to $K_3Fe(CN)_6$ concentration at a fixed NADH concentration (0.3 mM) has been determined for several preparations, and the reciprocal plots of such experiments are presented in Fig. 5. From these plots values of V_{max} and K_M were derived (Table VI). It can be seen that the

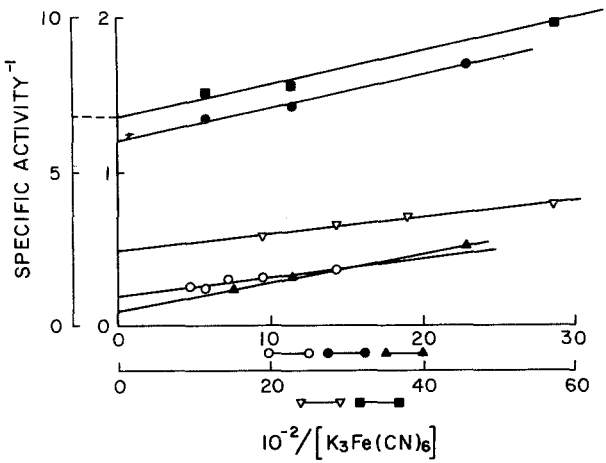

Figure 5. Reciprocal Plots of the NADH–$K_3Fe(CN)_6$ reductase activities of several cytochrome-*c* oxidase prepartions. Conditions: Assays were performed as described in text at a fixed concentration of 0.3 mM NADH. Curves: ▲—▲, preparation 1, 10 μg protein; ●—●, preparation 2, 70 μ protein; ■—■, preparation 3, 500 μg protein; ▽—▽, preparation 5, 72 μg protein; ○—○, preparation 7, 40 μg protein. Specific activity, μmoles $K_3Fe(CN)_6$ reduced/min/mg protein.

NADH–$K_3Fe(CN)_6$ reductase of preparation 1 has a maximal specific activity from one to two orders of magnitude greater than those of the other preparations. Thus, with preparation 1 as an example, it would seem that the Triton procedure for preparation of cytochrome oxidase is the least efficient in removal of NADH–$K_3Fe(CN)_6$ reductase. The same conclusion would also appear to hold for both the NADH–NBT and NADPH–NBT reductase (Table II). As with preparation 1, the V_{max} values of preparations 2–7 are uniformly low in relation to values found by others for purified mammalian NADH dehydrogenase [22, 23].

TABLE VI. Activity of NADH–$K_3Fe(CN)_6$ reductase contaminating cytochrome-*c* oxidase preparations (values obtained from the reciprocal plots of Fig. 5)

Preparation	Extrapolated maximal specific activity[a]	K_M(mM) [$K_3Fe(CN)_6$]
1	10.5	169
2	0.8	24
3	0.14	16
5	2.1	1.2
7	5.1	6

[a] μmoles $K_3Fe(CN)_6$ reduced/min/mg protein.

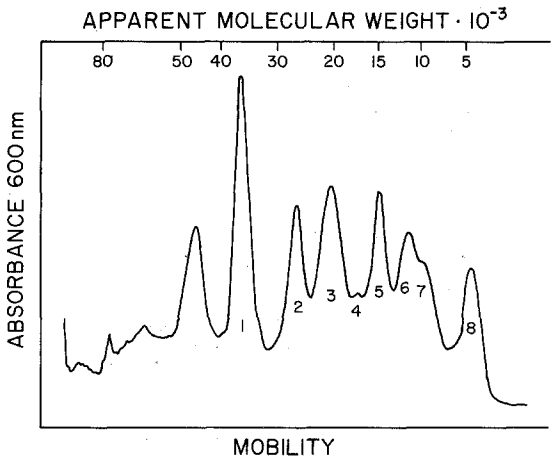

Figure 6. Determination of the contents of the NAD^+ elution of AGNAD following its prior equilibration with a cytochrome-*c* oxidase preparation. Conditions: The bed of AGNAD of the experiment of Fig. 1A was eluted with 3 ml 25 mM NAD^+, which was diluted, upon efflux, with phosphate–Tween; 100 μg of protein were subjected to electrophoresis.

Similarly, the K_M values for $K_3Fe(CN)_6$ as an electron acceptor are aberrant for most of the preparations, although it must be noted that the K_M values found for preparations 5 and 7 are in reasonable agreement with values of 4 and 5 mM which have been reported for solubilized NADH dehydrogenase preparations [22, 23]. It has not been determined whether the large value of K_M of preparation 1 might be attributable to residual traces of Triton detergents.

The ability of pyridine nucleotides to specifically elute certain reductases from AGNAD has allowed the putative identification of one of the reductases, namely NADH–NBT reductase. When an NAD^+ eluate of a bed of AGNAD (which had previously been equilibrated with cytochrome-*c* oxidase) is subjected to electrophoresis in the SDS–urea system, the 46,000 apparent formula weight component is found to have been specifically concentrated by the process of NAD^+ elution. A representative experiment is shown in Fig. 6. The densitometric scan shows that the 46,000 formula weight component of the original pattern of the oxidase preparation (Fig. 3) is still present, but its concentration in the eluate has been increased in relationship to the subunits of cytochrome-*c* oxidase. Thus, we conclude that the 46,000 formula weight component is the NADH–NBT reductase; the physical evidence of its concentration in Fig. 6 corroborates the enzymic evidence of Table V.

Beyond the foregoing, more detailed knowledge of the specific character of the dehydrogenases contaminating cytochrome-*c* oxidase preparations is limited. In isolated preliminary experiments, we have observed minimal rates of NADH–ubiquinone reductase when the uniquinone is dispersed in Azolectin, but we have found little or no demonstrable NADH–cytochrome-*c* reductase activity (in mM NaCN) and no detectable NADPH–$K_3Fe(CN)_6$ reductase. Further experiments to characterize the dehydrogenases are presently underway.

Discussion

It is well known that many of the less soluble components of mitochrondrial respiration and energy transduction are much alike in their solubility properties, relative hydrophobicities, etc. Several laboratories have found that cytochrome-*c* oxidase preparations of high purity may contain vestiges of the ATP synthetase [24–26]. Two reports of findings similar to ours should be noted. Tottmar and Ragan [27] found that both cytochrome-*c* oxidase and cytochrome-*b* copurified with NADH–$K_3Fe(CN)_6$ reductase in an extended series of steps used in the isolation of the latter enzyme from *Torulopsis utilis*. Likewise, in attempts to purify the terminal respiratory pigment, cytochrome-0 of *Vitreoscilla*, Webster and Liu [28] found it to be inseparable from an associated NADH–cytochrome-0 reductase. At best, Webster and Liu were only able to isolate chromatographic fractions which were relatively enriched in either NADH–cytochrome-0 reductase or cytochrome-0, respectively.

The Numbers and Amounts of Contaminating Proteins in Cytochrome-c Oxidase Preparations

Our findings indicate that at least two of the three contaminant reductases are separate entities. The NADH–$K_3Fe(CN)_6$ and NADH–NBT reductases can each be eluted from AGNAD under conditions where one is derived essentially free of the other. In addition, the fact that NADH–$K_3Fe(CN)_6$ reductase was copurified with cytochrome-*c* oxidase (Table III) attests to its nonidentity with the NBT reductases. At present we presume the NADH–$K_3Fe(CN)_6$ and NADH–NBT reductases to be derivatives of the NADH dehydrogenase of complex I. We have yet to define a condition wherein the NADPH–NBT reductase can be specifically eluted from a matrix in an improved state of purity or appreciable yield. However, it should be noted that beef heart submitochondrial particles have been found to exhibit substantial rates of oxidative phosphorylation, reversed electron flow, and transhydrogenase activity with NADPH as a substrate [29]. The dehydro-

genase which subserves these activities could well be the source of the NADPH–NBT reductase demonstrable in our experiments.

Our inability to accomplish a clean separation of cytochrome-*c* oxidase from the reductases on a large scale has prevented direct estimation of the contribution the contaminant proteins make to the total protein of the preparations. However, we have been able to make indirect estimates of this number for one preparation (#7 of Table I). One estimate of the percentage of contaminating protein has been made on the assumption that staining intensities obtained from densitometric scans are approximately equivalent to protein content. On this basis, we calculate from the upper densitometric scan of Fig. 3 that the components of greater than 40,000 apparent formula weight constitute 14% of the total protein. The significance of this value is enhanced by a second estimate which is obtainable from the plots of binding of heme aa_3 and protein of Fig. 4. In this experiment, at intervals 3–5, the binding of heme aa_3 declined to a minimum, as did that of protein. However, it can be seen that these two minima were not of the same numerical value. The binding of protein assumed an essentially constant percentage figure in excess of the extent of heme aa_3 binding. At intervals 3, 4, and 5 there was, respectively, 12%, 12%, and 14% more protein than heme aa_3 bound. We consider it significant that this number is essentially the same as that derived by densitometric analysis.

Saturation binding experiments such as that of Fig. 4 have not been performed with all of the preparations of Table I. However, densitometric scans of separations of preparations 3–6 upon electrophoresis in SDS–urea systems indicate that those preparations exhibit a range of from approximately 5% to 15% protein of apparent weights greater than 40,000. Such experiments establish also that preparations 3–7 each contain three contaminant species of 46,000, 52,000, and 65,000 apparent formula weights, and several preparations contain either one or both of two additional components of 70,000 and 78,000 MW [21].

Present evidence indicates that these components are not present as a consequence of artifacts of aggregation due to the temperature of dissociation. For example, preparation 7 contains minor amounts of four components of 46,000, 52,000, 65,000 and 78,000 (Fig. 3); we have found in control experiments that the absolute amounts and relative proportions of each (determined by Coomassie Blue staining [21]) are identical whether dissociation was performed under either our standard conditions or at 37° C for 3 hr. Minor amounts of proteins intrinsic to the inner membrane and complex IV with formula weights exhibiting close agreement with the foregoing have been reported previously [30, 31]. In addition, the NADH dehydrogenase resolvable from ETP of cardiac mitochondria has a reported formula weight of 70,000 Daltons [22]. In view of their binding by AGNAD it is possible to speculate that the observed family of contaminants of from 46,000 to 78,000 Daltons

are derivative forms (possibly both active and inactive) of the NADH and NADPH dehydrogenases of cardiac mitochondria. However, such an interpretation is unwarranted at present; the character of the electrophoretic separation of the contaminants has thwarted attempts to demonstrate activity of one type or another by either staining reactions or by spectrophotometry. The correlation of the 46,000 Dalton band with NADH–NBT reductase activity which we do establish (Table V and Fig. 6) indicates that, if that entity is a derivative of the original NADH dehydrogenase of cardiac mitochondria, it has either been substantially modified or it is a component subunit of the original enzyme. The latter possibility has been postulated for a component of complex I of 43,000 MW by Hare and Crane [31]. Work on further characterization of the reductases is continuing.

*The Binding of Cytochrome-*c *Oxidase by AGNAD*

Single sample equilibrations of cytochrome-*c* oxidase with AGNAD can accomplish clear separations (Fig. 1), although the losses of oxidase which are encountered are substantial. However, we have found no condition wherein the various reductases can be exclusively bound by AGNAD, or released therefrom, unaccompanied by any cytochrome-*c* oxidase. The binding of cytochrome-*c* oxidase by AGNAD can be presumed to be the composite result of at least two types of interaction. First, agarose matrices having spacer groups which connect the agarose and the functional moiety are known to exhibit hydrophobic interactions with proteins [32]. Indeed, some proteins are bound by matrices which simply bear unsubstituted spacer moieties [33, 34], and a procedure for the hydrophobic chromatographic purification of cytochrome-*c* oxidase of *Neurospora* has been reported [35]. In addition, agarose matrices which have been prepared by the use of CNBr for the coupling step possess substituted isourea and imidocarbamate moieties which are strongly basic [32]. Thus, the known affinity of cytochrome-*c* oxidase for cationic macromolecules, together with its essentially hydrophobic character, would render it susceptible to both types of binding upon equilibration with AGNAD.

Not surprisingly, several degrees of relative strength in the binding of cytochrome-*c* oxidase have been discerned upon its equilibration with AGNAD. There is a very weak binding which can be reversed upon washing the matrix with phosphate–Tween, although the bulk of the bound cytochrome-*c* oxidase cannot be so released. When such a washed matrix sample is subsequently treated with either NAD^+ or NAD(P)H to specifically elute one of the reductases, substantial amounts of cytochrome-*c* oxidase are also released. However, the largest part of the cytochrome-*c* oxidase bound by AGNAD is only released upon the

subsequent use of 5% SDS or a chaotrope such as perchlorate or guanidine hydrochloride. Thus, at least three magnitudes of binding strength are displayed by this sytem. The results of a recent experiment serve to exemplify these points. Of some 78 nmoles of heme aa_3 bound by a bed of AGNAD upon equilibration with preparation 7, successive elutions with NADPH (2×) and NAD^+ (2×) caused the release of 26%, 4%, 14%, and 1%, respectively, of the bound cytochrome-*c* oxidase. Minor amounts of cytochrome-*c* oxidase were also removed in interspersed washes with phosphate–Tween, but the bulk of the heme aa_3 (45%) was retained and was not released until the matrix bed was finally eluted with 5% SDS. In present experiments we are exploring the utility of the strong affinity of AGNAD for cytochrome-*c* oxidase as a potential means of large-scale preparation of the enzyme subunits.

The Effects of AGNAD Treatment on Cytochrome-c Oxidase Activity

The alteration in K_M for ferrocytochrome-*c* is the most interesting and consistent result of equilibrating cytochrome-*c* oxidase with AGNAD. The profound increases in rate constants for low substrate concentrations are consistent with the decreased values of K_M. However, it is apparent that treatment with AGNAD exposes a fundamental difference in the character of the preparations (# 5 vs # 7) that is not apparent on the basis of either the heme to protein ratio (Table I) or polypeptide composition. This difference (or differences) results in the V_{max} of one preparation being markedly enhanced by AGNAD treatment while that of a second preparation is slightly inhibited (Fig. 2).

At least three potential mechanisms can be advanced to explain how AGNAD can alter the kinetic characteristics of cytochrome-*c* oxidase: (1) The matrix could remove one or more less native species of cytochrome-*c* oxidase; (2) removal of the reductases could eliminate one or more species of same which inhibit cytochrome-*c* oxidase by virtue of protein–protein interactions; (3) removal of the reductases could facilitate a favorable conformation change in the enzyme.

In regard to the foregoing, it should be noted that neither preparation 5 nor preparation 7 yield any spectral evidence of the presence of denatured enzyme in the 422–424–nm region of reduced spectra—either before or after equilibration with AGNAD. In addition, analyses of the V_{max} of the cytochrome-*c* oxidase of eluates derived from AGNAD by the use of pyridine nucleotides establish that the presence of the enriched reductases therein are not inhibitory to the oxidase activity. Lastly, a preliminary experiment has established that the inhibitory action of ferricytochrome-*c* on the rate constant of preparation 7 is enhanced following its equilibration with AGNAD. It is apropos to note that Vanneste et al. [36] have demonstrated the accrual of an indeterminate number of inactive or latent species of cytochrome-*c*

oxidase during purification. The actual amount of such forms varied with the preparative procedure but they constituted a substantial part of the preparation in some instances. Our results provide corroborating evidence that cytochrome-*c* oxidase preparations must commonly contain one or more forms of diminished activity and that an equilibration with an NAD^+-affinity matrix corrects the circumstance. It is hoped that further experimentation will disclose their identity and character and the mechanism of their formation

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