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Discussion Letter

Heterogeneity in an isolated membrane protein

Has the 'authentic cytochrome oxidase' been identified?

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The criteria of homogeneity or native state of a protein are prone to become ambiguous when applied to membrane proteins, such as cytochrome-*c* oxidase, which are purified by extraction with detergents. Properties of the purified material depend on the detergent used and on details of the purification protocol followed with any single batch of a preparation. We present arguments to show that the evidence presently available in published form does not justify the designation [(1987) J. Biol. Chem. 262, 3160–3164] of one type of preparation as being closer to the native state than others.

X-ray absorption spectroscopy; EPR; Cyanide reactivity

1. INTRODUCTION

Cytochrome-*c* oxidase is one of the most intensively studied membrane proteins, a consequence both of its ubiquitous critical function in the cell's utilization of dioxygen and because of the challenges that it offers for investigators in a wide range of disciplines [1]. Its four functional metal components, each of which has unusual properties and makes unique contributions to catalysis, offer numerous 'handles' for imaginative experimentation. On many fronts, our knowledge of this enzyme has been pushed beyond that of other membrane proteins so that cytochrome oxidase is becoming a model for membrane proteins and for trying out new methodology to investigate them, similar to the role that hemoglobin plays for soluble proteins.

At the 1967 symposium on cytochromes convened by K. Okunuki at Kobe on the occasion of the 7th International Congress of Biochemistry in Tokyo [2], M.R. Lemberg, pioneer in heme chemistry and Chairman of the session on cytochrome-*c* oxidase at the symposium, said in his introductory remarks: "The 1964 symposium at Amherst [3] ended on a rather dismal note – it appeared that everybody's oxidase preparation was not only different, but also better than everybody else's. While we have not yet overcome this state of affairs entirely...there is nevertheless...justification for optimimism. Let us remember...that as

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Abbreviations: EXAFS, extended X-ray absorption fine structure; EPR, electron paramagnetic resonance

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much as our friends from protein chemistry and quantum physics can help us, they themselves do not yet have all the answers."

Have we overcome this state of affairs today? For those who thought so, a recent publication [4] was a sobering disappointment.

As in Lemberg's days, a variety of types of preparations and modifications thereof have been described and are in use in various laboratories around the world. It is now well recognized that not only are there differences among these various types of preparations, but there can also be differences between individual batches derived from the same preparative procedure which may be as extensive as those between different procedures [5–8]. There is as yet no single method that can reliably detect all differences that have been reported, although there are a number of tests that have been found useful in detecting the more obvious ones. One such test is the use of electron paramagnetic resonance (EPR) spectroscopy to detect the so-called '$g\sim12$' signal, a sign of a man-made irreversible alteration in the oxygen reduction site [8]. In the presence of NO or NO plus fluoride, EPR signals develop which diagnose the presence of particular conformational states [7]. One of the most decisive and readily applied tests is the determination of the kinetics of the reaction of the enzyme, as isolated, with cyanide [5,7,8]. There seems to be a relationship between cyanide reactivity and the $g\sim12$ EPR signal (vide infra).

At least among those active in the field there seemed to be agreement with the words of Naqui et al. [5] that "cytochrome oxidase...isolated by different methods, and by the same method at different times, is known to be heterogeneous. Such variability can be simply interpreted in terms of purified cytochrome oxidase being a variable mixture of more than one distinct molecular form of the enzyme". Naqui et al. [5] have also investigated a total of seven different preparations obtained from four basic preparative procedures using reactivity with cyanide as the probe of conformational heterogeneity. As published in 1984, this was an important contribution and we agree with the basic conclusions which were drawn. Since then, Baker et al. [8] have published a thorough investigation of the reasons for the heterogeneity as revealed by cyanide reactivity and came to the significant conclusion that enzyme reacting slowly with cyanide (the 'slow' form) shows generally poor reactivity with ligands and exhibits the $g\sim12$ EPR signal in proportion to the content of slow form in a given preparation. As far as is known to date, these properties are irreversible characteristics of a preparation; they are only temporarily reversed by a reduction-reoxidation cycle. Neither the $g\sim12$ EPR signal nor the slow form is found in mitochondria or submitochondrial particles (SMP), from which it seems to follow that the slow form and the $g\sim12$ EPR signal are signs of damage or at least modification to the enzyme. In all likelihood, these modifications occur in the immediate vicinity of the metal sites, predominantly at the oxygen reduction site (a_3Cu_B) [8].

2. DISCUSSION

With this background and particularly in view of the previous experimental results and statements of Naqui et al. [5], it was difficult for us to understand the logic behind the claim by Powers et al. [4] of having resolved "the conflicting results for the purified preparations by different methods", elevating the Yonetani (Y) type of preparation [9] to the "authentic cytochrome oxidase, if indeed any of the purified preparations are such", and specifically branding one alternative preparation, the Hartzell-Beinert (HB) preparation [10], as inferior. It is our view that the available experimental evidence does not support such a claim.

The basic argument made by Powers et al. [4] is that the Cu X-ray absorption spectrum of the enzyme isolated by the Y procedure [9] resembles that of the enzyme present in SMP, but does not resemble that of the enzyme isolated by the HB procedure [10]. The inference is that since the form of cytochrome oxidase in SMP is presumably the authentic form, then the enzyme isolated by the Y procedure (but not by the HB procedure) must also be the authentic form, having Cu sites that are structurally similar to those of cytochrome oxidase present in SMP.

In the study by Powers et al. [4], only figs 2 and 4 show direct comparisons between the two isolated HB and Y forms of the enzyme. The difference in the Cu EXAFS Fourier transforms (FTs) (fig.4 of [4]) is the appearance of a split first-shell peak for the enzyme prepared by the HB procedure

but not for the enzyme prepared by the Y procedure. Although this *may* indicate slight structural differences in the average copper site, one of us (R.A.S.) has shown [6] that differences of this kind are insignificant compared to the noise in the data and that the FT is an overly sensitive measure of EXAFS differences. We see variation in the splitting of this main FT peak among samples prepared by the same procedure in different laboratories [6]. Powers et al. [4] do not show a direct comparison of the Cu EXAFS data of the two isolated enzymes, which would have conveyed a more realistic picture of the extent of any differences. Fig.2 of the same article compares the Cu edges of various resting samples, indicating apparent differences in the height of the shoulder present at 8988 eV. These significant differences are not reproduced in studies by one of us (R.A.S.) on a variety of preparations of isolated cytochrome oxidase. All of the Cu edges we have observed (including data on samples prepared by each of the major isolation procedures) are similar to that of the sample prepared by the HB procedure exhibited in fig.2 of [4]. (One good measure of the height of the 8988-eV shoulder is the ratio of (F/I_0) at 8988 eV to that at the top of the edge (8997 eV). For ten recently examined samples, including samples HB/Ub and Y/E of [6], we observe this ratio to be in the range 0.56–0.58. For the data in fig.2 of [4], the sample prepared by the Hartzell-Beinert procedure exhibits a ratio of 0.57, while the sample prepared by the Yonetani procedure exhibits a ratio of 0.50.) This type of edge is more reminiscent of that reported for the pulsed state of the enzyme [4], an activated form which has been shown to exhibit only 'rapid' cyanide reactivity and no $g\sim12$ EPR signal [5,8].

Aside from the inconclusive nature of the evidence for significant XAS differences between different enzyme preparations, other striking inconsistencies exist in the argument presented by Powers et al. [4]. The Yonetani preparations were found to be 'highly homogeneous' in cyanide reactivity. The authors neglect to point out that they were homogeneous in having almost only *slow* form (>85%), in agreement with the later studies of Baker et al. [8]. They would thus be expected, as Baker et al. have shown, to have a large $g\sim12$ component, both properties now taken as a poor characteristic of a cytochrome oxidase preparation. While one might argue that the slow form is the species present in the membrane-bound enzyme, the authors' own data refute such an argument for they have included in their previous study [5] a preparation of membranous oxidase reported by Frey et al. [11]. This preparation showed 100% of the rapid form in the cyanide test in agreement with Baker et al. [8] who also find no $g\sim12$ EPR signal in this material. In contrast, of the soluble enzyme preparations examined by Naqui e al. [5], the lowest proportion (75 and 38%) of slow form was found with two preparations of the HB type [9,12], a preparation they consider inferior to the Y type of preparation. It is interesting to note that Baker et al. [8] actually find much higher percentages of rapid form (58–96%) in the six HB preparations they investigated and 100% rapid form in three modified HB type preparations. Baker et al. [8] point out that in the single sample of (unmodified) HB preparation examined by Naqui et al. [5], the amount of rapid form is abnormally low, and that this sample is therefore unlikely to be representative of enzyme prepared by the HB procedure. This sample is presumably the same sample examined by Powers et al. [4], although nowhere in [4] are the source, history and characteristics of the sample of the HB preparation used mentioned.

We have used the Hartzell-Beinert type of preparation for many years and believe that much useful information has come from this work. We know now that, unless special precautions are taken, this preparation may show heterogeneities like most or all other types of preparations [8]. We are keenly interested in learning of all available information on this enzyme, negative or positive. In view of the statements of Naqui et al. [5] quoted above and our present understanding of the problem of heterogeneity of isolated membrane proteins, however, we find it counter-productive when general conclusions are drawn concerning the properties and absolute quality of a preparation of this enzyme on the basis of a flawed study by a single technique of a single batch of an undefined preparation. We would expect adequate description of the material used and, if generalizations are to be made, adequate statistics. The problems we are facing with this type of enzyme are sufficiently complex by themselves, that sweeping and inaccurate generalizations of the kind we have de-

scribed [4] are only apt to complicate them further, particularly for the outsider or newcomer to this field.

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