

The Extinction Coefficients for Cytochrome aa_3 and their Consequences.

Yoshikawa and coworkers (Mochizuki *et al.* (1999) *J. Biol. Chem.* **274**, 33403-33411) have argued for a reevaluation of cytochrome *c* oxidase extinction coefficients, based upon a new metal analysis of their crystalline preparation. This also allowed a reinterpretation of the stoichiometries of aa_3 reduction and oxidation, in the light of the possible presence of a peroxide fragment in the heme pocket of the resting enzyme.

I have for many years used a value of 27 mM^{-1} as the "double difference" extinction coefficient for reduced *minus* oxidized, 605-630 nm, beef heart oxidase. This was based upon a relatively slow enzyme of the King-Kuboyama type. The value was determined using the original spectral data of Takashi Yonetani and the double difference method of Britton Chance to permit reliable determinations of concentration in all preparations including those with problems of light scattering and baseline changes upon oxidoreduction. A recent (Canadian, Brock) soluble preparation gave the following comparative coefficients at the two wavelengths concerned for cytochrome aa_3 (two hemes):

Dithionite reduced: at 605 nm: 43.3 mM^{-1} ; at 630 nm 4.8 mM^{-1} ;
Reduced difference (605-630nm): 38.5 mM^{-1} .
Oxidized (mixed fast/slow) at 605 nm 18.5 mM^{-1} ; at 630 nm 7.0 mM^{-1} ;
Reduced *minus* oxidized difference at 605 nm (red-ox): 24.8 mM^{-1} .
Overall double difference coefficient, 605-630 nm, red-ox, 27.0 mM^{-1} .

This is equivalent to 13.5 mM^{-1} per heme a group.

Soret extinction coefficients seem to be somewhat less reproducible (cf. oxidized maximum shifts, stray light errors, background scattering etc.). But the old 100 mM^{-1} (per heme) value of Yonetani for reduced enzyme - later reiterated by others (Caughey and Yoshikawa himself in *The Enzymes* 3rd. ed. Vol. XIII) - is surely too low. The alpha/Soret ratio should be 5.1 to 5.2. If the 604 nm value is 44-45 mM^{-1} , the absolute Soret value for reduced enzyme is therefore approx. 230 mM^{-1} (per aa_3) or half that per heme, say 115 mM^{-1} per heme group.

It may be noted that the difference between the differential contributions of *a* and a_3 at 605 nm (84% cyt. *a*, 16% cyt. a_3) would suggest absolute extinction coefficients of 30.0 mM^{-1} for *a* and 13.3 mM^{-1} for a_3 at 604-605 nm (if their oxidized forms have similar extinctions), consistent with the values seen in other single heme hemochromogen and pentacoordinate reduced species.

These values were checked by a atomic absorption metal analysis at Concordia University of a typical example of the same enzyme prepared at Brock University, which gave compositions very close to 3.0 Cu, 2.0 Fe, and 1.0 Mg per aa_3 . A detailed calculation using the Fe & Cu concentrations originally obtained by Steffens *et al.* (*Eur. J. Biochem.* **164** (1987) 295-300), assuming 3 Cu and 2 Fe per aa_3 , gives values of $24.8 \pm 1.49 \text{ mM}^{-1}$ (beef heart) and $23.4 \pm 1.03 \text{ mM}^{-1}$ (*Paracoccus*) for the difference extinction coefficient reduced *minus* oxidized at 604 nm (alpha maximum). The former value is identical to the Brock value. Steffens *et al.* (1987) themselves cited the 24.0 mM^{-1} value (12.0 mM^{-1} per heme) of van Gelder (1966) and did not propose the recalculation suggested by their own data - an increase of not more than 3%. Later (G.C.M. Steffens *et al.* (1993) *Eur. J. Biochem.* **213**, 1149-1157), they used a value of 25.3 mM^{-1} recommended by Vanneste (1966).

Hemochromogen analysis of the *a* heme may be unreliable but a combined *c:a* hemochromogen analysis of *B. subtilis caa₃* gave 1 heme *c*: 2 heme *a* and values for heme *a* close to those above (Assempour & Hill (1999) *Biochim. Biophys. Acta* **1320**, 175-187). Mochizuki *et al.* (1999) list a series of *E* values at 604-630 nm (red) ranging from a 43-year old Yonetani value of 33 mM⁻¹ to Vanneste's 1966 value of 38 mM⁻¹ which was based upon careful hemochromogen and CO binding measurements. The latter is essentially the same as that of 38.5 mM⁻¹ used for the Brock enzyme (above). In place of these values Mochizuki *et al.* (1999) propose one of 46.6 mM⁻¹, an increase of 21% over the previous quasi-consensus.

There are numerous different assays which confirm or employ the classical 24 or 25 mM⁻¹ (red-ox, 604/605nm), 38 mM⁻¹ (red, 604/605-630 nm), and 27 mM⁻¹ (red-ox, 604/605-630 nm) values. These have included (some more reliable than others):

- (i) hemochromogens (*aa₃* and *caa₃*);
- (ii) metal analysis;
- (iii) CO titration;
- (iv) HCN titration;
- (v) NADH/PMS redox titration;
- (vi) TMPD titration of *aa₃CN*;
- (vii) oxygen titration of the reduced form;
- (viii) tight cytochrome *c* binding;
- (ix) dithionite titration of the oxidized forms;
- (x) proton release &/or binding (e. g. Capitanio *et al.* (2000) *Biochemistry* **39**, 6373-6379).

All would need reevaluation if the classical values are >17% too low .

This also has chemical consequences because the number of redox equivalent-accepting or binding sites per heme are determined using a standard coefficient. Based upon the 46.6 mM⁻¹ value for 604-630nm (reduced) Mochizuki *et al.* find up to 6.4 one-electron reducible sites per *aa₃*. The conventional value (17% smaller) gives 5 one-electron sites. As the initial electron equivalent (or 0.8 electron equivalent) in their titration appears not to reduce a heme group (cf. their Fig. 5) the overall heme-linked stoichiometry is close to 4.0 with the classical coefficients and 5-6 with the higher value. NADH-PMS (as used by the Amsterdam group) gives smaller values for reducible sites (4.5 to 5.1 using the high extinction coefficient, or 3.7 to 4.2 using the classical value). The strong reductant dithionite may well be able to reduce sites in the enzyme preparation at a very negative redox potential that are not enzymatically significant. However a reevaluation by another research group might be useful.

This problem is linked to the question of the nature of the linking group found by X-ray crystallography, lying between the *a₃* heme and the CuB in pdb file 2occ representing a resting enzyme form. Yoshikawa *et al.* have identified this species as a peroxide, whose presence would indeed give rise to a requirement for 6 reducing equivalents to reform the fully reduced enzyme. However the relationship between this proposed species and the equivalent redox state 'P' form of the enzyme is unclear. But the 1.6 to 1.7 Å distance between the two centres and the asymmetry of the density make it difficult to ascribe it to a pair of hydroxyl groups or to a single atom of some kind. I have wondered about the possibility of a chloride ion in two alternate locations, one close to the *a₃* iron and the other close to the CuB. A chloride atom is about twice the size of an oxygen. This could be tested by preparing the oxidase in a specifically chloride bound form. The other species ligation of

which gives rise to a resting spectroscopic form of the enzyme is formate. This should give rise to a three-atom species in the heme pocket: "O=CH-O", probably associated with both Cu and Fe centres. Unfortunately we do not currently have an X-ray structure in the pdb for any formate-associated hemoprotein, although it is a readily accessible state at least of catalase (there are a couple of pdb structures with the larger acetate ion homologue present: lupin 1lh1 leghemoglobin acetate has Fe-bound acetate; human 1dgg catalase acetate is problematic - the acetate ion is nowhere near the Fe). We do need crystal structures of some more oxidase states as well as crystal structures of oxidized and reduced forms prepared from solution in different crystal forms (cf. T. Sjögren and J. Hajdu (2001) "The structure of an alternative form of *Paracoccus pantotrophus* cytochrome *cd*₁ nitrite reductase" J. Biol. Chem. 10.1074/jbc. M103657200 in press, on the hysteresis between redox states in cytochrome *cd*₁, *Paracoccus* nitrite reductase in the crystal state).

The possibility that there are significant differences between the spectra of the several *aa*₃ oxidases now available also needs checking by stoichiometric titrations of samples of such oxidases and the availability of fully reduced and oxidized spectra for examples of these enzymes. In particular complete and precise spectra for the reduced and oxidized *Paracoccus* and *Rhodobacter* enzymes would be useful, as well as comparative spectra for a typical *caa*₃ enzyme (*B. subtilis*, PS3 or *Thermus thermophilus*). Once checked they could perhaps be mounted on Graham Palmer's oxidase web site together with the beef heart values. Public availability of standard spectra should be as possible as of atomic coordinates. Possibly an evening session on these formal, important but notionally boring topics at the next full oxidase meeting would be justified.

Peter Nicholls.

24/July/2001