Vol. 114, No. 2, 1983 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 29, 1983 Pages 822-828

PREPARATION OF MONOMERIC CYTOCHROME C OXIDASE: ITS KINETICS DIFFER FROM THOSE OF THE DIMERIC ENZYME

Katarzyna A. Nałęcz*, Reinhard Bolli and Angelo Azzi

Medizinisch-chemisches Institut der Universität Bern
Bühlstrasse 28, CH-3012 Bern, Switzerland

Received June 3, 1983

Bovine cytochrome c oxidase in 0.1% dodecylmaltoside, 50 mM KCl and 10 mM Tris-HCl, pH 7.4 is monodisperse with an apparent M_r 360,000 (dimer) as estimated by filtration on Ultrogel AcA 34. In the absence of added KCl the apparent M_r is 160,000 (monomer). The dimeric enzyme has a high and a low affinity site for cytochrome c; the monomeric, only the high affinity site. The results are consistent with the existence of one active site per monomer, having high affinity for cytochrome c. Since in a dimer the two sites are in close proximity, the binding of the first molecule of cytochrome c to the first site hinders the binding of the second molecule to the second site. The kinetic data fit with a model of homotropic negative cooperativity. The effect of salts on the cytochrome c oxidase kinetics is also present in isolated bovine heart mitochondria.

Cytochrome c oxidase from bovine heart (EC 1.9.3.1) contains up to 13 polypeptides (1). Depending on their M_r and number a minimum M_r for the monomeric enzyme can be estimated between 160,000 and 200,000. The enzyme can exist in different states of aggregation, but the nature of the minimum functional unit has been a matter of discussion (2). Indications that the monomers of cytochrome c oxidase can be active came recently from several groups (3-5). The kinetic behaviour of these enzyme species has not however been studied. Two binding sites have been described for cytochrome c on native cytochrome c oxidase, the first of low molecular activity and high affinity, the second of high molecular activity and low affinity (6).

Both sites have been suggested to be catalytically competent (7), or one site only, the low affinity site having a regulatory function (8) and no connection with the proton translocation reaction (9).

*Permanent address: Nencki Institute of Experimental Biology, Warsaw, Poland

0006-291X/83 $1.50

In this study we describe a simple and mild technique to produce monomers of bovine heart cytochrome c oxidase, by dispersing the enzyme in dodecylmaltoside in the absence of salts. It was found that the monomers possess only one phase of high affinity in the saturation kinetics with cytochrome c while the dimers possess two, of high and low affinity. The cytochrome c oxidase activity of bovine heart mitochondria has the same salt dependence of the isolated enzyme, suggesting that in the native membrane monomerisation of cytochrome c oxidase can take place.

MATERIALS AND METHODS

Cytochrome c type VIII (Sigma), dodecylmaltoside (Calbiochem), cholate (Fluka), Ultrogel AcA 34 (LKB) were used in this study. Mitochondria from bovine heart were prepared according to (10) and kept frozen. Before use, they were thawed and washed either in 250 mM sucrose, 10 mM Tris-HCl pH 7.4 or in 150 mM sucrose, 10 mM Tris-HCl pH 7.4 and 50 mM KCl. Cytochrome c oxidase was prepared according to Yu et al (11) Apparent M_r were estimated by determining the protein hydrodynamic radius relative to a set of standards (ferritin, catalase, IgG and aldolase) using an Ultrogel AcA 34 column (1x45 cm) equilibrated with the detergent dodecylmaltoside (0.1%) in 10 mM Tris-HCl, pH 7.4 (5) in the presence or absence of 50 mM KCl. Protein concentration was estimated by using the biuret method (12). The activity of cytochrome c oxidase was assayed spectrophotometrically (7) in an Aminco DW-2a spectrophotometer at 550-540 nm using the extinction coefficients 19.4 $mM^{-1}.cm^{-1}$ and 24 $mM^{-1}.cm^{-1}$, reduced minus oxidised for cytochrome c and cytochrome c oxidase, respectively. Integrations, curve-fitting and simulations were carried out in Commodore 2001 and 8032 computers equipped with a BBC-Servogor analog plotter.

RESULTS

The isolation of monomers and dimers by Ultrogel AcA 34 chromatography is shown in Fig. 1. The enzyme diluted 10-folds in 0.1% dodecylmaltoside in a buffer containing 10 mM Tris-HCl pH 7.4 in the presence and absence of 50 mM KCl was applied to the column and eluted with the same buffer with or without 50 mM KCl. In presence of KCl cytochrome c oxidase was eluted in a sharp peak centered at fraction 20, in the absence of salts around fraction 26. The elution pattern was highly reproducible. The first protein species corresponded to an apparent M_r of 360,000, the second to an apparent M_r of 160,000 consistent with a monomer and a dimer of cytochrome c oxidase, respectively.

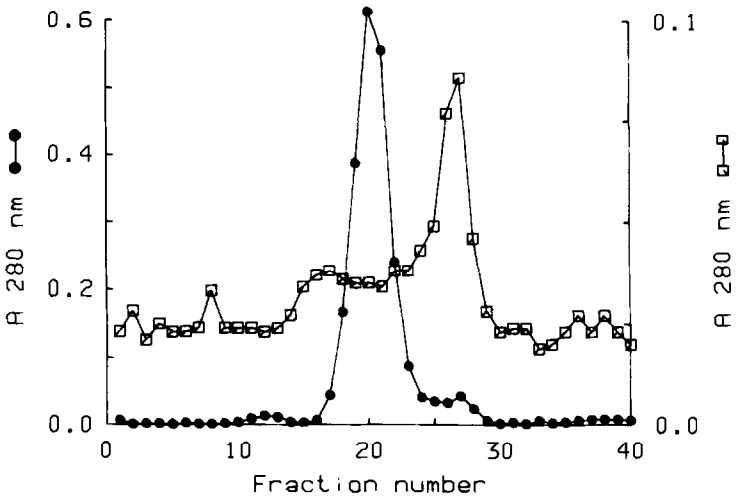

Figure 1. Gel filtration on Ultrogel AcA34 of cytochrome c oxidase at low and high ionic strength. Cytochrome c oxidase (1.5 nmol) after 10-fold dilution in 10 mM Tris-HCl, pH 7.4, and incubation in presence (●) or absence (□) of 50 mM KCl for 2 hours) was loaded onto an Ultrogel AcA 34 column (45 x 1 cm). The elution profile in the absence of salt shown in the figure was obtained after rechromatography of the monomers isolated in a first run.

Polyacrylamide gel electrophoretic as well as spectroscopic analyses of the fractions, not reported here, indicated that both fractions contained a complete, native cytochrome c oxidase. The retardation of the cytochrome c oxidase elution in the absence of KCl was not an artifact due for instance to the interaction of the protein with the column. At increasing salt concentrations the position of the elution of monomers and dimers remained the same while the relative proportions of monomers and dimers changed (Fig.3). Morover, under conditions in which the dimers did not dissociate (Triton X-100), they were eluted (not shown) in the absence of KCl without retardation. The kinetics of the enzyme in low and high salt concentrations were studied and are reported in Fig. 2A in form of an Eadie-Hofstee plot. At 50 mM KCl two phases existed one of high affinity and low molecular activity, a second of low affinity and low molecular activity. At low salt concentration only one phase is observable having high affinity for cytochrome c and low molecular activity. The data plotted according to the Hill equation (13) have consistently given for the high salt con-

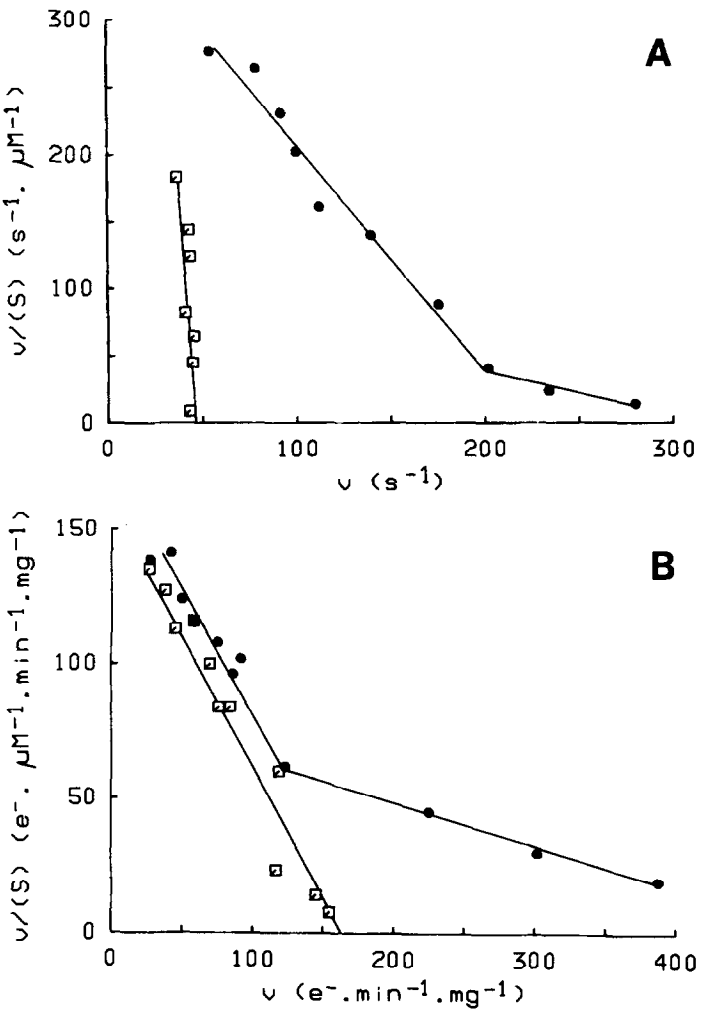

Figure 2A. Eadie-Hofstee plots of the activity of native cytochrome c oxidase at high and low ionic strength. The activity was measured spectrophotometrically as described in the Methods in 10 mM Tris-HCl, pH 7.4 and 0.1% dodecylmaltoside. The concentration of reduced cytochrome c was 0.2-20 μM, that of cytochrome c oxidase 0.6 nM. (□) no KCl, (●) 50 mM KCl. The molecular activity (V) corresponds to nmol cytochrome c oxidised per second per nmol heme aa_3.

Figure 2B. Eadie-Hofstee plots of the activity of cytochrome c oxidase in bovine heart mitochondria. Conditions as in Figure 2A except that 0.25 and 0.1 M sucrose was added in the absence and presence of KCl, respectively, to maintain the osmotic pressure constaht. The concentration of mitochondrial protein was 5.2 μg/ml. (□) no KCl, (●) 50 mM KCl.

ditions a Hill coefficient around 0.5. In the absence of KCl the Hill coefficient was always close to 1. In the presence of intermediate salt concentrations (Fig.3) the Hill coefficient varied between 1 and 0.5,

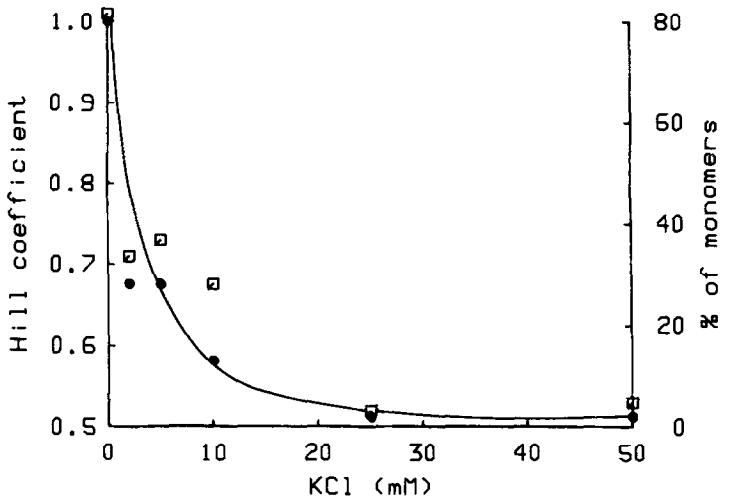

Figure 3. Comparison between the cytochrome c oxidase monomers and Hill coefficient at different salt concentrations. The conditions for the gel filtration from which the proportion of monomers (●) was calculated, were described in the legend of Fig. 1 and those for the experiments from which the Hill coefficient (◻) was calculated in the legend of Fig. 2.

going from 0 to 50 mM KCl, parallel to the proportion of monomeric complexes of cytochrome c oxidase. In Fig. 2B bovine heart mitochondria were used which in the absence of salt exhibited only one phase of high affinity (Hill coefficient = 1.0), while in the presence of 50 mM KCl two phases were distinguishable (Hill coefficient = 0.7).

DISCUSSION

The experiments reported above were carried out using bovine heart cytochrome c oxidase in monomeric or dimeric form. It was found that the enzyme, usually a dimer, under very low ionic strength conditions became monomeric. The kinetics of the two species were different, the dimers having a biphasic saturation behaviour, the monomers a monophasic one. The dissociation of the dimers induced by a low salt concentration was found to be fully reversible (not shown). After chromatography in Ultrogel AcA 34 at low salt concentration, the monomer of cytochrome c oxidase had monophasic kinetics in low salt (Hill coefficient = 1.0) and polyphasic kinetics (Hill coefficient less than 1) in 50 mM KCl. A description of the cytochrome c oxidase in

which two non-interacting binding sites exist in each monomer of cytochrome c oxidase is not adequate to explain the steady state kinetic behaviour of this enzyme. The findings described above are consistent with the following model. Cytochrome c oxidase dimers contain two cytochrome c binding sites, namely one per monomer, located in the cleft created by the two adjacent monomers (4). In each molecule of cytochrome c the area of the exposed heme edge, containing lysyl 13 residue, interacts with subunit II of one cytochrome c oxidase monomer. The opposite side of the cytochrome c molecule interacts with subunit III of the other monomer (14). The dimension of the cleft between two monomers of cytochrome c oxidase in which cytochrome c binding occurs cannot exceed approximately 4-5 nm, the transverse diameter of the complex. It follows that, in a dimer, the occupation by one molecule of cytochrome c (2-3 nm) of one binding site will (for its geometry (15) and its electrical dipole (16)) hinders the occupation by the second molecule of the second site. Such a molecular interaction leads to an apparent negative cooperativity, which is observed in the dimers but not in the monomers of the enzyme. The half-of-the-sites reactivity which has been also described for the binding of cytochrome c to cytochrome c oxidase (17) is an extreme expression of the negative cooperativity. Such an interaction between neighbouring molecules of cytochrome c creates the conditions for increasing the off-rate constant of cytochrome c (rate limiting) with consequent increase in the V_{max}, as can be seen in Fig. 2 A and B, at high salt concentrations, i.e. in dimers. The off-rate constant, smaller in the monomer, is responsible for the high affinity of cytochrome c and the low molecular activity of this cytochrome c oxidase species. The type of detergent, the ionic strength, and other situations, still under investigation, are responsible for the transition between monomers and dimers of cytochrome c oxidase. Such a transition is accompanied by a decrease in the molecular activity and by an increase in affinity for

the substrate. It is worth mentioning that at low salt concentrations the vectorial proton translocation catalysed by cytochrome c oxidase is absent (9), an event which may be related to the monomerisation of the enzyme. On the basis of the data presented here it is interesting to speculate that the different kinetic behaviours of cytochrome c oxidase obtained in the presence of different types of phospholipids and at different concentrations of salts are due to different proportions of aggregates, dimers and monomers of the enzyme under those conditions. Also in vivo the activity of cytochrome c oxidase may be regulated by the same parameters.

ACKNOWLEDGEMENTS

We like to thank Dr. Ganka Kossekova for the very stimulating discussions during the initial part of this work. The financial support of the Schweizerischer Nationalfonds (N.3.739-0.80) of the Sandoz Stiftung and of the Emil-Barrel Stiftung is gratefully acknowledged.

REFERENCES

1. Kadenbach, B., Jarausch, J., Hartman, R. & Merle, P. (1983) Analyt. Biochem., 129, 517-521.
2. Azzi, A. (1980) Biochim. Biophys. Acta 594, 231-252.
3. Darley-Usmar, V.M., Alizai, N., Ayashi, A.I., Jones, G.D., Sharpe, A. & Wilson, M.T. (1981) Comp. Biochem. Physiol. 68B, 445-446.
4. Capaldi, R.A., Darley-Usmar, V., Fuller, S. & Millett, F. (1982) FEBS Lett. 138, 1-7.
5. Rosevear, P., Van Aken, T., Baxter, J. & Ferguson-Miller, S. (1980) Biochemistry 19, 4108-4115.
6. Ferguson-Miller, S., Brautigan, D.L. & Margoliash, E. (1976) J. Biol. Chem. 251, 1104-1115.
7. Ferguson-Miller, S., Brautigan, D.L. & Margoliash, E. (1978) J. Biol. Chem. 253, 149-159.
8. Fuller, S.D., Darley-Usmar, V.M. & Capaldi, R.A. (1981) Biochemistry 20, 7046-7053.
9. Wikström, M. & Pentilla, T. (1982) FEBS Lett. 144, 183-189.
10. Smith, A.L. (1967) Methods Enzymol. 10, 81-86
11. Yu, C., Yu, L. & King, T.E. (1975) J. Biol. Chem. 250, 1383-1392.
12. Gornall, A.G, Bardawill, Ch. & David, M.M. (1949) J. Biol. Chem. 177, 751-766.
13. Dixon, M. & Webb, E.C. (1979) in Enzymes (Longman, London) p. 424.
14. Birchmeier, W., Kohler, C.E. & Schatz, G. (1976) Proc. Natl. Acad. Sci. U.S.A. 73, 4334-4338.
15. Dickerson, R.E. and Timkovich, R. (1975) in The Enzymes (Boyer, P.D. ed.) vol. 11, pp. 397-547, Academic Press, New York
16. Koppenol, W.H. & Margoliash, E. (1982) J. Biol. Chem. 257, 4426-4437.
17. Bisson, R., Jakobs, B. & Capaldi, R.A. (1980) Biochemistry 19, 4173-4178.