Biochemical Pharmacology, Vol. 33, No. 1, pp. 109–113, 1984.
Printed in Great Britain.

0006-2952/84 $3.00 + 0.00

EFFECT OF BEPRIDIL ON THE ACTIVITY OF CYTOCHROME *c* OXIDASE IN SOLUTION AND IN PROTEOLIPOSOMES

ERALDO ANTONINI, MAURIZIO BRUNORI, ALFREDO COLOSIMO and PAOLO SARTI*

Institute of Chemistry and Biochemistry, C.N.R. Centre of Molecular Biology, Faculty of Medicine, I University of Rome (La Sapienza), Rome, Italy; and Department of Biochemistry, Faculty of Medicine, II University of Rome (Tor Vergata), Rome, Italy

(*Received* 17 *March* 1983; *accepted* 29 *July* 1983)

Abstract—The interaction between bepridil and mitochondrial cytochrome *c* oxidase has been studied using the purified enzyme either in aqueous suspensions in the presence of detergents, or embedded into phospholipid vesicles. The investigation, systematically extended to nonactin and valinomycin for comparison, showed that: (a) valinomycin and nonactin induce similar changes in the visible absorption spectrum of cytochrome oxidase; these changes are quite different from those induced by bepridil. (b) The three compounds have an effect on the functional properties of purified, solubilized oxidase which may be related to binding. In particular, bepridil displays a complex pH-dependent effect which at concentrations below 50 μM results in a stimulation of the activity of approximately 30% starting with the oxidized resting enzyme. At variance with valinomycin and nonactin, the stimulatory effect is the same, within the errors, for the detergent-suspended, the vesicle-embedded and even the Keilin–Hartree particles. (c) In the case of detergent-suspended oxidase, the stimulatory effect of bepridil is also similar whether the enzyme is in the resting or in the pulsed state. If the oxidase is embedded into vesicles, however, the pulsed state is significantly more sensitive to bepridil than the resting one. These results are discussed in the light of the possible role assigned to pulsed oxidase in the regulation of the electron flux through the respiratory chain.

Bepridil is a newly synthesized drug whose antianginal and antiarythmic effects have been related primarily to an inhibitory action on Ca^{2+} binding sites of the cardiac sarcolemma [1–3]. Another relevant effect of bepridil involving mitochondrial oxidative phosphorylation has been investigated by Younes *et al.* [4]. These authors reported that indeed bepridil affected oxidative phosphorylation, acting as an uncoupler on the FAD-linked substrates but not on the NAD-linked substrates. No significant effect of bepridil on the activity of cytochrome *c* oxidase was reported.

In this paper we present a re-investigation of the effect of bepridil on cytochrome *c* oxidase, using either the detergent solubilized enzyme or the enzyme embedded into phospholipid vesicles.

Throughout the investigation, nonactin and valinomycin, two well-known ionophores [5], have been confronted with bepridil with regard to the spectroscopic and the functional changes induced on the oxidase. This approach is useful not only in providing additional information relevant to the interpretation of the pharmacological effects of bepridil, but also in yielding new results on the catalytic mechanism of the enzyme, especially in connection with the relationships between different states of the oxidase [6] and the phospholipid environment.

* To whom correspondence should be addressed.

† Abbreviations: bepridil, 1-[3-isobutoxy-2-(phenylbenzyl)amino]propyl pyrolidine; HEPES, *N*-2-hydroxyethylpyperazine-*N'*-2-ethane sulfonic acid; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenyl-hydrazone; RCR, respiratory control ratio.

MATERIALS AND METHODS

Chemicals. Sodium ascorbate and HEPES† were from BDH; bepridil was obtained from Ravasini-Organon (Rome, Italy); cytochrome *c* (type VI), nonactin and valinomycin were from Sigma Chemical Co. (St. Louis, MO); asolectin was from Associated Concentrates (Woodside, Long Island, NY) and was partially purified before use according to Hinckle *et al.* [7]. Emasol was from Kao-Atlas (Tokyo, Japan). All other chemicals were of analytical grade.

Enzyme preparations. Beef heart cytochrome *c* oxidase was purified following the procedure of Yonetani [8]. Keilin–Hartree particles were prepared according to King method 1 [9] as modified by Ferguson-Miller *et al.* [10].

Oxidase inserted into liposomes was obtained following the methods described in the literature [7, 11]. Each preparation was submitted to tests for respiratory control ratio and catalytic activity, as previously described [12].

Spectrophotometric and stopped-flow measurements were carried out, respectively, on a thermostated Cary 219 instrument and on a Durrum–Gibson apparatus equipped with a 2 cm observation chamber (dead time ~4 msec).

The concentration of the haemoproteins was determined spectrophotometrically using the extinction coefficient of the reduced form, i.e. values of 27.6 mM^{-1}/cm at 550 nm for reduced cytochrome *c* and 21 (total haem) mM^{-1}/cm at 605 nm for reduced cytochrome oxidase, respectively [8].

Cytochrome c *oxidase activity* was determined spectrophotometrically in air following the oxidation

of cytochrome *c* reduced with stoichiometric sodium ascorbate. Assays were routinely run in 50 mM HEPES + 50 mM KCl, pH 7.0. For soluble oxidase, 0.1% emasol v/v was usually present in the medium. In order to give statistical relevance to activity determinations, under some critical experimental conditions, several assays in the presence and absence of effectors were carried out in succession.

Absorption changes upon addition of effectors were followed by differential spectroscopy using a Cary 219 instrument.

RESULTS AND DISCUSSION

Spectral changes

Addition of bepridil, nonactin and valinomycin to the oxidized resting (R_o) state of cytochrome oxidase in detergent (emasol) caused a pertubation in the Soret region of the spectrum. Figure 1 shows the difference spectra obtained for these three compounds and also reports (for comparison) the difference spectrum observed in going from the pulsed to the resting state ($P_o \rightarrow R_o$) of cytochrome oxidase [13], which is clearly different. It can be seen from Fig. 1 that, while the difference spectra observed for nonactin and valinomycin are the same, that obtained upon addition of bepridil is characteristically different in amplitude and shape (although with the same isosbestic point at 420 nm).

The titration reported in Fig. 2A shows that the apparent dissociation constant for bepridil (K_d *ca* 130 μM) is much greater than that of either nonactin or valinomycin (K_d *ca* 10 μM). Moreover, it was observed that the formation of the complex between bepridil and oxidase was inhibited by emasol. Thus, in going from 0.15% to 1% emasol the total absorbance change decreases by a factor of approximately 2. This effect is also illustrated in Fig. 2B, which shows the dependence on emasol concentration of the amplitude of the spectral perturbation (425–410 nm) at a fixed concentration of bepridil (i.e. 500 μM).

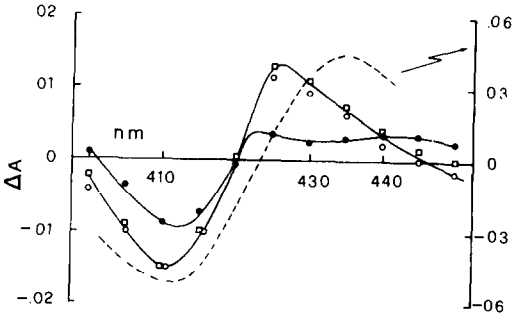

Fig. 1. Changes in the Soret absorption band of cytochrome *c* oxidase induced by various effectors. The difference spectra were run using 1 cm light path cuvettes and 5 μM cytochrome *c* oxidase in 50 mM HEPES (pH 7) containing 0.1% emasol and 50 mM KCl. The concentrations of the effectors were: 507 μM for bepridil (●); 117 μM for both nonactin (□) and valinomycin (○). The temperature was 20°. The dashed line refers to the difference spectrum between the pulsed and resting forms of the enzyme recorded under identical conditions for the completely oxidized species ($A_{P_o} - A_{R_o}$).

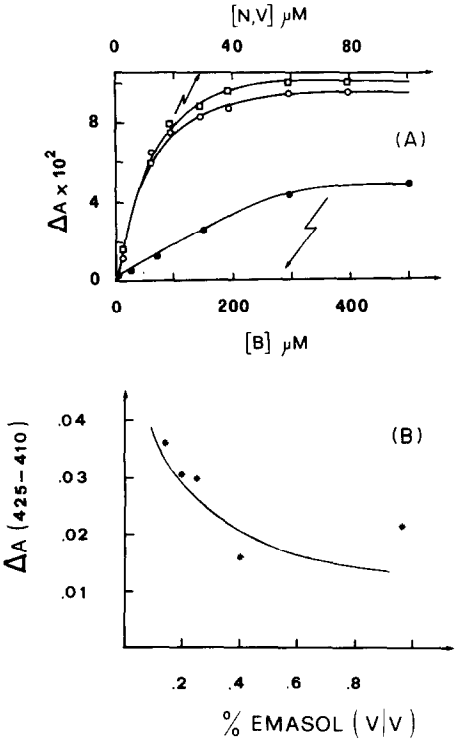

Fig. 2. Spectrophotometric titrations of soluble cytochrome *c* oxidase with various effectors. (A) Absorbance changes resulting from the addition of increasing amounts of bepridil (●), nonactin (□) and valinomycin (○) to a solution of 20 μM oxidized resting cytochrome *c* oxidase, recorded as differences in absorption at two wavelengths (i.e. 425–410 nm in the case of bepridil and 427–410 nm in the case of valinomycin and nonactin). All experimental conditions were as in Fig. 1. (B) Dependence of emasol concentration on the maximum absorbance change observed in the presence of 500 μM bepridil.

Similar experiments carried out with oxidase in solution in the presence of dithionite ($Na_2S_2O_4$) indicated that the addition of bepridil produced a small spectral perturbation of the Soret band of the enzyme; this effect, however, was not observed with either nonactin or valinomycin.

Experiments carried out with oxidized resting (R_o) oxidase inserted into liposomes yielded a somewhat different behaviour. While valinomycin and nonactin still perturbed the spectrum of oxidase with an apparent dissociation constant K_d *ca* 10 μM (Fig. 3), no effect was observed upon addition of bepridil up to a concentration of 100 μM.

These spectral data indicate: (i) that the three effectors all bind to solubilized cytochrome oxidase in the oxidase resting state (R_o), inducing a characteristic spectral perturbation; (ii) that the apparent dissociation constant is considerably greater for bepridil compared to that for nonactin and valinomycin; and (iii) that the presence of detergent—and especially of a phospholipid membrane—seems to depress considerably the effect observed with bepridil; in particular, no spectral perturbation was observed with oxidase inserted into liposomes.

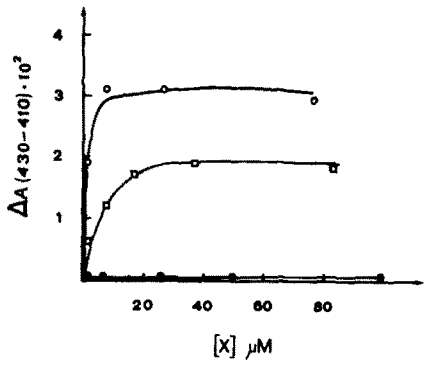

Fig. 3. Spectrophotometric titration of the interaction between cytochrome *c* oxidase embedded into liposomes and various effectors. Bepridil (●), valinomycin (□) and nonactin (○) were added to a suspension of proteoliposomes containing 10 μM oxidase in 50 mM K^+ HEPES + 50 mM KCl, pH 7.0. The temperature was 20°.

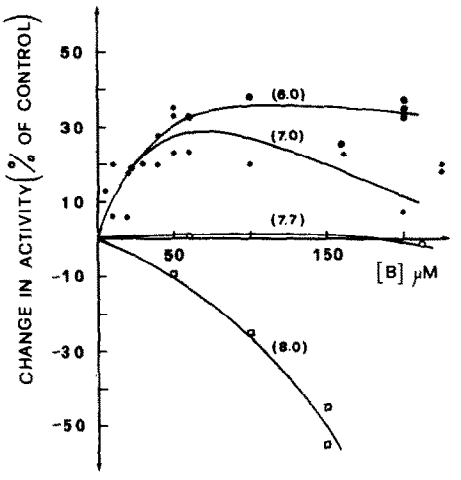

Fig. 4. Effect of bepridil on the activity of soluble cytochrome *c* oxidase as a function of pH. The initial rate of oxidation of reduced cytochrome *c* (21 μM) by cytochrome *c* oxidase (150 nM) in the presence of different amounts of bepridil was recorded spectrophotometrically at 550 nm at different pH values. All experiments were carried out in air. In all cases the buffer was 50 mM K^+ HEPES containing 0.1% emasol and 50 mM KCl; the temperature was 20°. The ordinate refers to the percentage change in the rate of oxidation of reduced cytochrome *c* compared to the corresponding value measured in the absence of effectors.

Functional data

The effect of bepridil, nonactin and valinomycin on the rate of oxidation of cytochrome *c* in air was investigated with oxidase in detergent and in the liposomes. Table 1 summarizes some of the results obtained under different conditions.

With oxidase in detergent the effectors displayed a complex behaviour. At a very low emasol concentration (i.e. 0.003%), bepridil and nonactin stimulated the rate of cytochrome *c* oxidation in the lower concentration regime while they inhibited activity at higher concentrations (*ca* 100 μM). This inactivation has been partly related to precipitation of the oxidase (and thus to effective reduction of the total enzyme) upon addition of increasing concentrations of bepridil and nonactin, as shown by (a) the presence of turbidity in the sample and (b) the reduction of this inhibitory effect upon increase in emasol concentration (e.g. up to 0.1%).

The results obtained with bepridil in 0.1% emasol at four pH values are reproduced in Fig. 4 in terms of the change in enzyme activity as a function of total bepridil concentration. Examination of the data indicates that activation is only observed at pH 6 and 7, while at pH 8 only inhibition is apparent. This complex behaviour is the result of two opposing effects related to activation and inhibition which, under some conditions (e.g. pH 7.7), tend to cancel each other.

Table 1. Effect of bepridil, nonactin and valinomycin on the activity of cytochrome *c* oxidase

Assay medium	pH	% Increase (bepridil)	% Increase (nonactin)	% Increase (valinomycin)	State of oxidase
0.1% Emasol in 50 mM HEPES + 50 mM KCl	6.0	37 ± 5	40 ± 8	25 ± 10	Soluble, resting
	7.0	25 ± 7	24 ± 4	32 ± 12	Soluble, resting
	7.7	Ø	20	—	Soluble, resting
	8.0	Ø	29	30	Soluble, resting
	7.0	19 ± 1	25 ± 5	28 ± 5	Soluble, pulsed
50 mM K^+ HEPES + 50 mM KCl	7.0	32 ± 7	100 ± 10	87 ± 4	Vesicle-embedded, resting
	7.0	29	—	—	Vesicle-embedded, resting*
	7.0	67 ± 3	155 ± 5	110 ± 5	Vesicle-embedded, pulsed
	7.3	37	72	—	K–H particles, resting

* Stopped-flow experiments.

The uncertainty intervals represent maximum errors calculated over at least three independent experiments. Stock solutions of 4 mM bepridil in water and 2 mM nonactin and valinomycin in 95% ethanol were prepared immediately before use and added to the reaction mixture up to a final concentration corresponding to the saturation levels indicated in Figs. 4 and 5. K–H, Keilin–Hartree.

The stimulation of the activity of soluble oxidase, observed in the lower concentration regime (<50 μM), was investigated in considerably greater detail and a comparison of the results obtained with bepridil, nonactin and valinomycin is reported in Table 1. The maximum increase in activity was 37% (at pH 6.0), which is a small but not negligible effect. The result is statistically significant, as indicated by the reproducibility of the phenomenon.

Examination of the data allows the following conclusions: (i) the magnitude of the stimulatory effect starting with the resting (R_o) solubilized enzyme is similar for all three effectors, and amounts to approximately 30% at pH 7.0; (ii) the stimulation reaches a plateau, indicating that the effect is related to binding. In fact, it is interesting to note that in the case of nonactin and valinomycin the effect is titrated at or below 10 μM, while for bepridil this phenomenon is saturated at or below 50 μM; (iii) the effect of pH on the stimulation is difficult to assess in view of the overlap with the pH-dependent inhibition described above.

The action of bepridil, nonactin and valinomycin was also investigated for oxidase embedded into liposomes. Some of the results obtained with proteoliposomes characterized by an RCR of 3:4 are summarized in Fig. 5 and Table 1. It can be seen that upon addition of bepridil the activity of the resting enzyme is stimulated up to 30% at *ca* 50 μM; thus the effect of bepridil is similar to that observed for the enzyme in detergent, although no spectral perturbation was observed (see above). An experiment carried out by stopped-flow at higher concentrations of enzyme also showed an increase (30%) in the rate of cytochrome *c* oxidation (see Table 1) similar to that observed by spectrophotometry at much lower concentrations.

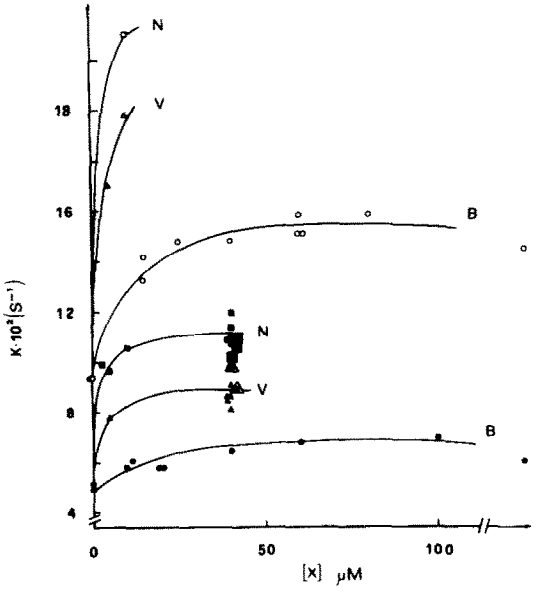

Fig. 5. Influence of various effectors on the catalytic activity of cytochrome *c* oxidase embedded into liposomes. Closed and open symbols refer, respectively, to the resting and pulsed forms of the enzyme, which in all cases was 70 nM. Circles, triangles and squares refer to bepridil, valinomycin and nonactin, respectively. The enzymatic activity is expressed as a pseudo-first-order constant for the oxidation of 22 μM cytochrome *c* in 50 mM K^+ HEPES, pH 7.0 containing 50 mM KCl. The temperature was 25°. Framed symbols refer to experiments in which either nonactin or valinomycin were added to liposomes already containing bepridil at a concentration of 40 μM.

Addition of nonactin and valinomycin has a considerably greater stimulatory effect, as expected on the basis of their activity as ionophores, catalysing the collapse of the ionic gradient across the membrane [5].

To clarify the effect of bepridil on the activity of proteoliposomes, we carried out experiments with the resting enzyme in which more than one effector was simultaneously present in the assay. The results are shown in Fig. 5. The addition of nonactin and valinomycin to vesox already containing saturating bepridil (i.e. 40 μM) brought about the same total increase in activity observed upon addition of the ionophores by themselves. Therefore these experiments show that the effects of the two ionophores (nonactin and valinomycin) and that of bepridil are not additive. It is not unlikely that bepridil, being a weak acid with some hydrophobic character, may act as a protonophore in the liposome system, possibly somewhat similar to FCCP.

A series of experiments to assess the effect of bepridil on the activity of pulsed cytochrome oxidase are also shown in Fig. 5. The more active state of the oxidase was also stimulated by bepridil, and the stimulatory effect was higher (67%) than that observed with the resting state when the enzyme was in the liposomes. We have recently proposed [12] that pulsed oxidase, the more active state of the enzyme, plays a role in the regulation of the electron flux through the respiratory chain. A possible physiological significance of the transition between resting and pulsed oxidase is being tested with experiments which should assess the state of the prevailing form of the enzyme *in vivo*. In this perspective, the finding that bepridil displays a greater stimulatory effect on pulsed cytochrome oxidase may be of significance in the interpretation of the action of this drug. In view of the significance that we attribute to the properties of pulsed oxidase, we carried out one set of experiments at different concentrations of bepridil using proteoliposomes characterized by an RCR of 8:9. We confirmed that bepridil exerts a stimulatory effect which leads to an increase in activity of 70% in the case of pulsed oxidase. However, we also found that the maximum activity increase was achieved at a drug concentration that was 4 to 5 times lower than that obtained for proteolyposomes with a lower RCR. Thus at a concentration of 10 μM bepridil displays a significant effect on the activity of pulsed oxidase.

Concluding remarks

We have shown that the addition of bepridil to solubilized cytochrome oxidase brings about an increase in activity in the concentration range below 50 μM and at a nmole drug/mg protein ratio of approximately 5×10^3. This increase in activity is possibly related to a direct binding of the drug to the enzyme, as shown by the appearance of spectral changes induced in the oxidized resting (R_o) state

of the oxidase, although at a lower value (*ca* 230) for the above ratio. Also nonactin and valinomycin seem to bind to resting oxidase, and both bring about an increase in enzyme activity similar to that observed with bepridil. The mechanism underlying this increase in activity is unknown, but it is unlikely to involve a stabilization of the more active pulsed (P) state of the enzyme in view of the character of the spectral perturbation (Fig. 1).

The effect of bepridil on oxidase activity is also observed when the enzyme is inserted into liposomes at a nmole drug/mg lipid ratio of 2.5×10^3. In this case, however, it cannot be excluded that the increase in activity is partly related to the possible action of bepridil as a protonophore, leading to a mechanism of activation similar to that attributed to some well-characterized uncouplers [5]. If this was the mechanism, it is difficult to involve the oxidase in the increased oxidative phosphorylation, reported by Younes *et al.* [4] as a remarkable effect of bepridil on entire mitochondria. The same conclusion was reached by these authors although the bepridil effect they observed was seen at a lower drug/total protein concentration (=50 in comparable units) than that reported here. This should not be taken as a serious limitation, however, since (i) the experimental systems studied in the two cases were extremely different and the drug/total protein ratio obviously acquired a different meaning when applied to either the entire mitochondrion or to the purified cytochrome oxidase; (ii) the two functional activities studied were also different, i.e. oxidative phosphorylation for the entire mitochondrion and electron transfer for the purified oxidase, being in the latter case the conclusion regarding oxidative phosphorylation only an indirect one. A direct comparison of our results with those reported by Younes *et al.* [4] is difficult at present. This is largely related to the fact that obviously the extent of the stimulatory effect exerted by bepridil depends on the extent of integration of the system, as well as the functional state of the oxidase. This is clear from our own experiments on liposomes, in which (as given above) the effect of bepridil is exerted at lower concentrations with the tighter vesicles. Thus, using proteoliposomes with an RCR of 8:9, the stimulatory action of bepridil is exerted at a concentration of 10 μM, which is compatible with the pharmacological dosage.

By referring to a two-state model proposed by us [14] to account for the mechanism of action of cytochrome *c* oxidase, the finding that the stimulatory effect of bepridil is significantly more marked when the experiments are carried out starting with pulsed oxidase in liposomes acquires some significance. Thus it may be proposed that, at an integrated level, the action of bepridil on cytochrome oxidase (and particularly on the pulsed state) may promote an easier flux of electrons through the enzyme, which is relevant to the pharmacological effects of the drug [2–4]. An understanding of the action of this drug on the function of cytochrome oxidase in the reconstituted liposome system (which mimics satisfactorily, but is not identical to the mitochondrion) may demand more sophisticated experiments, in which the rate of oxidation of cytochrome *c* and the rate of proton consumption and proton pumping are simultaneously determined in the transient time regime. These experiments are presently being initiated in our laboratory.

REFERENCES

1. D. Cosiner, P. Duchene-Marulloz, G. Rispat and G. Strichenberger, *Archs int. Pharmacodyn. Ther.* **225**, 133 (1977).
2. S. Vogel, R. Crompton and N. Sperelakis, *J. Pharmac. exp. Ther.* **210**, 378 (1979).
3. C. Labrid, A. Grosset, G. Dureng, J. Mironneau and P. Duchene-Marullaz, *J. Pharmac. exp. Ther.* **3**, 546 (1979).
4. A. Younes, N. Moins and M. Habert, *Biochemie* **59**, 73 (1977).
5. A. Lenhinger, *Biochemistry*, 2nd edn. Academic Press, New York (1979).
6. M. Brunori, E. Antonini and M. T. Wilson, in *Metal Ions in Biological Systems* (Ed. H. Sigel), Vol. 13, p. 187. Marcel Dekker, New York (1981).
7. P. C. Hinckle, J. J. Kim and E. Racker, *J. biol. Chem.* **247**, 1338 (1972).
8. T. Yonetani, *J. biol. Chem.* **235**, 845 (1960).
9. T. E. King, *Methods Enzymol.* **10**, 202 (1967).
10. S. Ferguson-Miller, D. L. Brautigan and E. Margoliash, *J. biol. Chem.* **251**, 1104 (1970).
11. J. M. Wrigglesworth, *Proc. 11th FEBS Meet.* **45**, 95 (1978).
12. P. Sarti, A. Colosimo, M. Brunori, M. T. Wilson and E. Antonini, *Biochem. J.***209**, 81 (1983).
13. M. Brunori, A. Colosimo, G. Rainoni, M. T. Wilson and E. Antonini, *J. biol. Chem.* **254**, 10769 (1979).
14. M. T. Wilson, J. Peterson, E. Antonini, M. Brunori, A. Colosimo and J. Wyman, *Proc. natn. Acad. Sci. U.S.A.* **78**, 7115 (1981).