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Metachromatic effects and binding of organic cations to energized submitochondrial particles

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SUMMARY

Energization of submitochondrial particles results in a marked increase of binding, measured as number of sites and binding constants, of the cationic dyes Acridine Orange and Neutral Red. The binding of the dyes is accompanied by spectral changes which are identified as metachromatic effects. The findings are interpreted in terms of interaction with electron-negative sites and stacking of the dye molecules.

Energization results in increased binding of anionic organic molecules in submitochondrial particles and decreased binding in intact mitochondria[1-3]. The binding has been interpreted as being due to electrostatic forces and taken to indicate an inversion of electrical polarity of submitochondrial particles with respect to intact mitochondria[1-3]. On the other hand, energization of submitochondrial particles results also in increased binding of Neutral Red and in a parallel spectral shift which has been identified as a metachromatic effect[4,5]. The spectral shift has been interpreted as being due to stacking phenomena of the dye following an enhanced electrostatic interaction with nucleophilic sites formed during energization. A dye which is always positively charged at neutral pH and well studied as titrator of the anionic sites is Acridine Orange. The present paper contains some data on the interaction of Acridine Orange and Neutral Red with submitochondrial particles which are relevant to the problem of the polarity of submitochondrial particles and of the conformational changes of the membrane during energization.

Liver and beef heart mitochondria were prepared by standard procedures. Submitochondrial particles from beef heart mitochondria were prepared by sonication in 5 mM $MgCl_2$, 2 mM ATP, and under N_2. Spectrophotometric measurements were carried out either with a double beam or with a split beam. The binding of Acridine Orange and Neutral Red to the particles was measured as described previously[4]. For the problems

Abbreviations: Hepes, *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulphonic acid; FCCP, carbonyl cyanide *p*-fluorometoxyphenylhydrazone.

related to the absorption of the dyes on the glass surfaces, the indications of Lamm and Neville[8] were followed. Acridine Orange and Neutral Red were recrystallized as described by Pal and Schubert[9].

Fig. 1 shows the absorption spectrum of Acridine Orange in the presence of submitochondrial particles. The spectrum was identical to that of the free dye in the case of the deenergized particles, indicating that passive interaction did not enhance the self-association of the dye molecules. On the other hand, addition of ATP resulted in a marked decrease of the α band of the dye. A similar effect was observed when energization was carried out through the addition of succinate or NADH. Subsequent deenergization of the particles by addition of FCCP resulted in a restoration of the initial spectrum. Similar changes of intensity of the α and β bands are obtained when Acridine Orange was bound to different types of polyanions.

Metachromatic effects were also obtained when the concentration of the free dyes, either Acridine Orange or Neutral Red, was increased. However, the metachromatic effects obtained with the energized particles were similar to those induced by the polyanions rather than to those occurring at high dye concentrations. The differences were more marked in the case of Neutral Red than in the case of Acridine Orange.

Fig. 2 shows the Scatchard plot for the binding of Acridine Orange to submitochondrial particles. Consider first the energized state. Three parts of this plot can be distinguished. At low dye to protein ratios, the ratio bound to free increases with the amount of bound dye. The cooperative aspect of the binding, which will be dealt with more extensively in forthcoming papers, indicates a perturbation of the membrane, accompanying the dye–membrane interaction, with unmasking of new binding sites. At intermediate dye to protein ratios, the plot shows sites with very

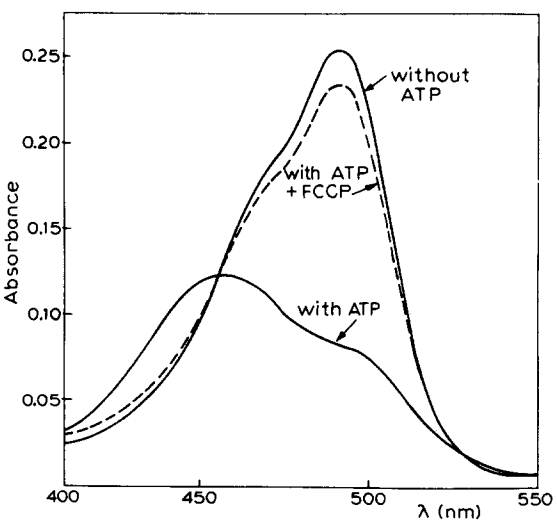
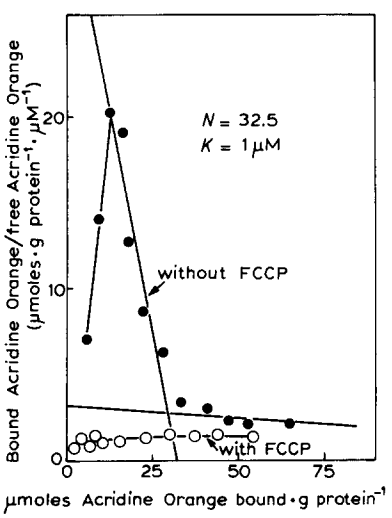


Fig. 1. Absorption spectrum of Acridine Orange in presence of energized and deenergized particles. The incubation medium contained in 2.5 ml, 0.1 M KCl, 2.5 mM $MgCl_2$, 10 mM *N*-2- hydroxyethylpiperazine *N*-2-ethanesulphonic acid (Hepes)–HCl, pH 6.7, 5 μM Acridine Orange, and 0.35 mg protein of beef heart submitochondrial particles. Energization was induced by the addition of 400 μM ATP and deenergization by 2 μM FCCP.

high affinity. The number of sites in this experiment was about 33 μmoles · g protein^{-1} with a dissociation constant of 1 μM. At high dye to protein ratios, the large dimension of the binding and the very low affinity are in accord with a process of dye–dye interaction. Fig. 2 shows also that when the particles were in the deenergized state there was a very limited binding and there were no high-affinity sites.

Similar phenomena were observed also with Neutral Red. Binding sites were about 150 μmoles · g protein^{-1}. A strict correlation was found between metachromatic effects and high-affinity binding sites. In fact, inhibition of the high-affinity binding sites with the local anesthetic nupercaine resulted in a parallel abolition of the metachromatic effect.

The spectral shift due to binding of Acridine Orange to polyanions is dependent on the ratio between number of dyes molecules and of binding sites[10]. The maximal effect is obtained when the ratio is close to 1. When the number of sites exceeds that of dye molecules, the redistribution of the dye on the polyanion chain results in a gradual decrease of the spectral shift[10]. Fig. 3 shows a spectrophotometric titration of the ATP-induced metachromatic effect carried out by maintaining the dye concentration constant and by increasing the amount of particles. The extent of ATP-induced change in absorbance at 492 nm (which reflects the metachromatic shift) increased with the increase in amount of protein until a maximum was reached and then tended to decrease. Extrapolation of the initial part of the plot permits the dye to protein ratio for maximal effect to be calculated, and therefore the number of binding sites per g protein. In this experiment the number was about 40 μmoles · g protein^{-1}.



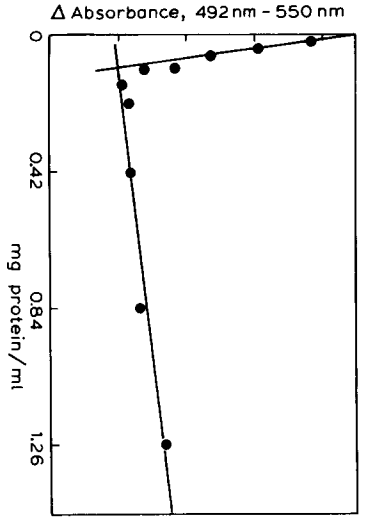


Fig. 2. Scatchard plot for the binding of Acridine Orange to energized and deenergized particles. The medium contained in 2.5 ml, 0.1 KCl, 10 mM Hepes–HCl, pH 7.0, 2.5 mM $MgCl_2$, 1 mM ATP, 2 mM succinate and 3 μM FCCP in the deenergized state. 0.75 mg mitochondrial protein. Particles prepared from beef heart mitochondria by sonication for 20 sec.

Fig. 3. Spectrophotometric titration of binding sites for Acridine Orange in energized particles. The medium contained in 2.0 ml, 0.1 M KCl, 10 mM Hepes–HCl, pH 6.5, 2.5 mM $MgCl_2$. The absorbance change, measured as ΔA (492 nm–550 nm) was induced by the addition of 500 μM ATP. The amount of protein in each experiment is indicated on the abscissa.

It might be suggested that the accumulation of the cationic dyes in the energized particles is similar to the uptake of weak acids in mitochondria which occurs at the expense of a pH gradient. An accumulation of free dye inside the particles is in contrast with the spectral data referred to above. Furthermore, such a mechanism requires the internal to external dye ratio to be equal to the ΔpH. Since the former may reach values of about 10^4, the ΔpH would be of 4 units and the internal pH below 3.No indication of such internal pH has been obtained.

We therefore conclude that energization of submitochondrial particles results in a marked increase of binding of organic cations. The process is proposed to reflect an electrostatic interaction with electron-negative sites which favour stacking of the dye.

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REFERENCES

1 A. Azzi, *Biochem. Biophys. Res. Commun.*, 37 (1969) 254.
2 L.L. Grinius, A.A. Jasaits, T.U.P. Kadziauskas, E.A. Liberman, V.P. Skulachev, V.P. Topali, L.M. Tsofina and M.A. Vladimirova, *Biochim. Biophys. Acta*, 216 (1970) 1.
3 E.A. Liberman and V.P. Skulachev, *Biochim. Biophys. Acta*, 216 (1970) 30.
4 R. Colonna, P. Dell'Antone and G.F. Azzone, *FEBS Lett.*, 10 (1970) 13.
5 G.F. Azzone, R. Colonna, P. Dell'Antone and S. Massari, *Colloq. on Bioenergetics*, Adriatica, Bari, 1971, in the press.
6 V. Zanker, *Z. Physik. Chem.*, 199 (1952) 225.
7 A.L. Stone and D.F. Bradley, *J. Am. Chem. Soc.*, 83 (1961) 3627.
8 M.E. Lamm and D.M. Neville, *J. Phys. Chem.*, 69 (1965) 3872.
9 M.K. Pal and M. Schubert, *J. Am. Chem. Soc.*, 84 (1962) 4384.
10 A.L. Stone and D.F. Bradley, *Biochim. Biophys. Acta*, 148 (1967) 172.