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Inhibition of the sarcoplasmic reticulum Ca^{2+}-ATPase by Reactive Red 120

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The triazine dye, Reactive Red 120, was found to bind tightly (K_d = 30) nM) and with low stoichiometry to sarcoplasmic reticulum membranes. Our finding that this high-affinity binding caused noncompetitive inhibition of the Ca^{2+}-ATPase indicates that the dye-binding site is distinct from both the active site and putative regulatory site. Detergent solubilization (monomerization) of the Ca^{2+}-ATPase caused a 25-fold decrease in affinity for Reactive Red 120, while causing no decrease in affinity toward another dye, Reactive Blue 2. For the Reactive-Red-120-inhibited enzyme, the level of steady-state enzyme phosphorylation by ATP was not significantly different from that exhibited by the control Ca^{2+}-ATPase. The rate of dephosphorylation in the presence and absence of ADP, however, was markedly decreased by the presence of the inhibitor. Distance measurements by fluorescence energy transfer from the active (FITC-reactive) site to the Reactive Red 120 site gave a value of 59 Å. Similar experiments yielded an average distance of 35 Å between the latter site and the tryptophan residues, most of which are postulated by the 'sequence model' (MacLennan et al. (1985) Nature 316, 696–700) to be located in a transmembrane domain.

Introduction

The sarcoplasmic reticulum (SR), an intracellular organelle, has as its function the control of cytosolic calcium levels in muscle cells. Release of calcium from the SR causes contraction of myofibrils; relaxation occurs upon subsequent sequestering of cellular calcium into the SR via active transport by an intrinsic enzyme, the Ca^{2+}-ATPase. Isolated as vesicles with the Ca^{2+}-ATPase active site outwardly oriented, skeletal SR is one of the simplest in vitro active transport systems. The Ca^{2+}-ATPase has been the subject of a large number of studies (for reviews, see Refs. 1 and 2), so that many steps in the catalytic cycle and numerous structural features, including the complete amino-acid sequence [3], have been elucidated. Nevertheless, much of the relationship between its structure and function remains unknown.

One of the more fruitful approaches to studying the structure and mechanism of an enzyme has been to use noncovalent probes. Among numerous examples of their use are the interaction of ouabain with the catalytic subunit of the $(Na^+ + K^+)$-ATPase, and aurovertin with the β subunit of

The abbreviations used are SR, sarcoplasmic reticulum, Mops, 4-morpholinepropanesulfonic acid, EGTA, ethylene glycol bis(2-aminoethyl ether) *N,N,N′,N′*-tetraacetic acid, Ado*PP*[NH]*P*, adenyl-5′-yl imidodiphosphate, SDS, sodium dodecyl sulfate, $C_{12}E_9$, poly(oxyethylene) 9-lauryl ether, FITC, fluorescein isothiocyanate (isomer I), I_{50}, concentration of inhibitor causing 50% inhibition, FET, fluorescence energy transfer, EP, phosphorylated enzyme intermediate

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mitochondrial [4] and bacterial [5] F_1-ATPases. Discoveries along these lines continue to be valuable, as is illustrated by the recent description of diethylstilbestrol as an F_0-directed ligand [6]. Such molecules can be used to reveal and characterize unique sites on the enzyme, inhibit one or more catalytic steps, or, in conjunction with fluorescent ligands, measure intersite distances by fluorescence energy transfer. The present study characterizes the interaction of such a probe, Reactive Red 120, with the SR Ca^{2+}-ATPase. This dye, when attached to agarose, was previously used to fractionate the SR proteins [7]. Reactive Red 120 is shown to bind strongly and with low stoichiometry to SR membranes. Dye binding causes noncompetitive inhibition of the Ca^{2+}-ATPase, indicating that its site is distinct from both the active site and the putative regulatory site. FET measurements suggest that the Reactive Red 120 site is distant from the active (FITC-reactive) site, perhaps being located close to the protein/phospholipid interface. Detergent solubilization dramatically lowers the affinity toward Reactive Red 120, illustrating that the probe is sensitive to the state of the enzyme in the membrane.

Materials and Methods

Sarcoplasmic reticulum vesicles were obtained from rabbit hind leg white skeletal muscle by the method of Eletr and Inesi [8]. Reactive Red 120, $C_{12}E_9$, FITC, rabbit muscle lactate dehydrogenase type II, rabbit muscle pyruvate kinase type II and fatty-acid-free bovine serum albumin were from Sigma; Reactive Blue 2 (Cibacron blue F3GA) was purchased from Polysciences. Reactive Red 8 was from Aldrich; all other chemicals were reagent grade or better. FITC-modified SR (5 ± 0.5 nmol FITC attached per mg SR protein) was prepared by a previously described procedure [9].

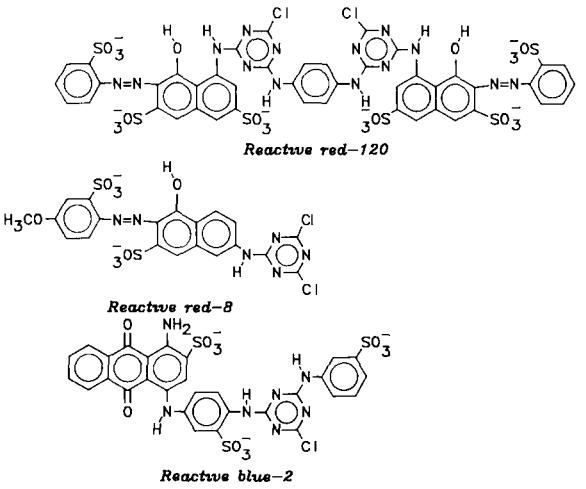

Fig 1 Structures of triazine dyes

Gel electrophoresis. SDS tube gel electrophoresis was performed according to the method of Laemmli [10] using a 5% stacking gel and a 12% running gel. A gel scanner attachment with a GCA-MacPherson spectrophotometer was used to monitor the migration of dyes; the wavelengths used in this procedure were: 495 nm (FITC); 525 nm (Reactive Red 120); and 595 nm (Bromphenol blue). To test for covalent labeling of SR proteins by dyes, the gels were fixed overnight with a trichloroacetic acid/methanol solution and were subsequently destained with 40% methanol/7% acetic acid.

Kinetic assays. All kinetic determinations were performed at 37 °C in solutions containing 80 mM KCl, 50 mM Mops (pH 7), 5 mM $MgCl_2$, 1.1 mM $CaCl_2$ and 1 mM EGTA plus other stated components. For studying the concentration dependence of inhibition of SR vesicular Ca^{2+}-ATPase by triazine dyes, the phosphomolybdate assay was used with 1 mM ATP and 0.04 μg of ionophore A23187 per μg of SR. The experimental procedure was that described in Coll and Murphy [11]. For other studies a coupled enzyme assay was used [12]. The inhibition of lactate dehydrogenase ($5 \cdot 10^{-9}$ g/ml) was studied in the presence of 1 mM dithiothreitol and 1 mM pyruvate. Initial velocities were determined spectrophotometrically at 340 nm with NADH varied between 4 μM and 300 μM.

The reversibility of Reactive Red 120 inhibition of the Ca^{2+}-ATPase was tested by incubating 2 mg/ml SR vesicles with 25 μM dye at room temperature in assay buffer. Aliquots of this mixture containing 10 μg of SR were then assayed for activity by the coupled enzyme procedure with 2.5 mM ATP and 0.4 μg of ionophore A21387. After recording the velocity of the dye-inhibited enzyme, 5 mg/ml (final) bovine serum albumin was added to the assay and the resulting increased rate was recorded.

For studies concerning the inhibition of solubilized Ca^{2+}-ATPase, the following procedure was used. SR vesicles (2 mg/ml final) were solubilized in a solution containing the following final concentrations: 20 mg/ml $C_{12}E_9$, 20% (w/v) sucrose, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 50 mM Mops (pH 7). Aliquots of this enzyme stock were then assayed with the coupled enzyme method using 10 μg SR and 2.5 mM ATP, with the final concentration of detergent adjusted by addition of a $C_{12}E_9$ stock solution. The activity of the enzyme was determined using several concentrations of pyruvate kinase and lactate dehydrogenase to ensure full coupling. The concentration of free dye was determined under each condition from the iterative program utilizing the NADH concentration and the dissociation constants and stoichiometries of dye binding to the coupling enzymes (Table I). The dye-inhibited velocities collected at each [detergent] were then treated by nonlinear regression analysis (see below) to yield values for the K_i of the soluble enzyme and the CE_{50} (concentration of detergent at which the concentrations of free and detergent-bound are equal). The parameters for interaction of pyruvate kinase and lactate dehydrogenase with dyes were determined spectrophotometrically; 5 μM dye was titrated with varying concentrations of enzyme and the resulting absorbance changes were analyzed by Eqn. 3. The absorption coefficient of lactate dehydrogenase used was $\epsilon_{280} = 162 \cdot 10^3\ M^{-1} \cdot cm^{-1}$ [13] and 35 000 subunit molecular weight was assumed. The absorption coefficient of pyruvate kinase used was $E^{1\%}_{280} = 5.4$ [14] and a subunit molecular weight of 57 000 was assumed.

Measurements of phosphorylated enzyme intermediate. The phosphorylation reaction was studied at 0 °C in an assay solution containing final concentrations of: 1.1 mM $CaCl_2$, 1.0 mM EGTA, 5 mM $MgCl_2$, 80 mM KCl, 50 mM Mops (pH 7.0) and 35 μM [γ-^{32}P]ATP. The reaction was initiated by addition of 0.2 mg/ml (final) SR vesicles containing 40 μg ionophore A23187/mg protein. Controls were obtained with reactions containing the above components in the absence of calcium. For studies concerning the time-dependence of phosphoenzyme concentration, time zero was taken as addition of EGTA; at the desired time intervals a 900 μl aliquot of the reaction was quenched with 200 μl of ice-cold 25% (w/v) trichloroacetic acid. Collection of precipitated SR protein and its treatment were carried out as previously described [7]

Triazine dyes. Reactive Red 120 stock solutions were prepared in doubly distilled water using the manufacturer's estimate of dye content (60% by weight, the remainder being NaCl). The resulting solution gave a absorption coefficient at 515 nm of 52 350 $M^{-1} \cdot cm^{-1}$. Reactive Red 8 stock solutions were prepared with the estimated dye content of 50% by weight; the resulting absorption coefficient at 550 nm was 39 600 $M^{-1} \cdot cm^{-1}$. The concentration of Reactive Blue 2 was determined using an absorption coefficient of 13 600 $M^{-1} \cdot cm^{-1}$ at 610 nm [15]. The partitioning of dyes into micelles was determined spectrophotometrically using the same experimental conditions as the kinetic assays. Scans of the visible spectra of 5 μM dye solutions were collected at several concentrations of $C_{12}E_9$. The data were analyzed as ΔA vs. $\Delta A/([C_{12}E_9] - \text{cmc})$; the slope of this plot was termed $-CE_{50}$.

Measurements of fluorescence quenching by Reactive Red 120 The fluorescence of unmodified SR vesicles (excitation, 290 nm; emission, 350 nm) and FITC-labeled SR vesicles (excitation, 490 nm; emission, 520 nm) was monitored at 25 °C with a Perkin-Elmer MPF-44B spectrofluorimeter using 10 nm slits for both excitation and emission. To a 2.5 ml assay solution identical to that used in the kinetic studies (except that calcium was omitted) were added sequential increments of dye. The recorded fluorescence at each dye concentration was corrected for inner filter effects and dilution. The data were then treated as described in the Data analysis section.

It should be noted that an artifact observed primarily at low SR concentrations had to be addressed in order to gain accurate and reproducible data. Briefly, it was found that at low SR concentrations mere manual stirring of the solution would produce a decrease in fluorescence. No evidence could be found to indicate that this was a vessel wall effect, and after several remixings a stable signal was obtained. Either the data were collected from an assay solution whose initial fluorescence signal had been stabilized by remixing, or the portion of fluorescence quenching due to

mixing was determined by sequential additions of aliquots of water and subtracted from the triazine dye data. Both methods gave the same results.

The distance, R, between donor and acceptor was calculated according to Fairclough and Cantor [16]:

$$R = (1/\mathrm{Eff} - 1)^{\frac{1}{6}} R_0$$

where Eff is the transfer efficiency, and R_0 is the Förster critical distance. The latter was obtained by.

$$R_0 = 0.979(\kappa^2 n^{-4} \phi J)$$

where κ^2 is the dipole orientation factor (assumed to be 0.67; [16]), n is the refractive index (assumed to be equivalent to that of alanine, i.e., 1.4), ϕ is the quantum yield of the donor in the absence of the acceptor, and J is the spectral overlap integral. The quantum yield for the tryptophan residues was determined to be 0.17 by the ratio method using tryptophan at pH 7 ($\phi = 0.13$) as a standard [17]. For FITC-modified SR, a value of 0.6 was used [9]. Values for J were calculated with the aid of a hand calculator as described by Fairclough and Cantor [16]; for the fluorescein-reactive red pair, we obtained a value of $3.4 \cdot 10^{11}$ $\mathrm{\AA}^3 \cdot \mathrm{M}^{-1}$; for tryptophan-reactive red, the value of J was $4.3 \cdot 10^{10}$ $\mathrm{\AA}^3 \cdot \mathrm{M}^{-1}$. The method was checked by calculating J for $1,N^6$-ethenoADP and NBD-tyrosine [18]; the results agreed within 1%. The value of ϵ at 515 nm for Reactive Red 120 bound to SR was found to be 52400 $\mathrm{M}^{-1} \cdot \mathrm{cm}^{-1}$.

Data analysis. Since many of the data collected in this study involve conditions in which free [ligand] is considerably less than total added [ligand], the results had to be treated in a manner accounting for this ligand depletion. Briefly, equations relating the observed dependent variable (enzyme velocity, v, absorbance difference, ΔA, or fluorescence intensity, F) to the concentration of free enzyme, [E], were derived by combining mass conservation equations with the equilbrium equation:

$$K = \frac{[\mathrm{L}][\mathrm{E}]}{[\mathrm{EL}]}$$

where [L] and [EL] are the concentrations of free dye and enzyme-dye complex, respectively, and K is the dissociation constant.

Solving for [E], one obtains the quadratic equation.

$$[\mathrm{E}] = 0.5([K + [\mathrm{L}]_t - BP]^2 + 4KBP)^{\frac{1}{2}} + BP - K - [\mathrm{L}]_t \qquad (1)$$

where $[\mathrm{L}]_t$ is the total dye concentration, P is the protein concentration, and B is the stoichiometry ($\mathrm{nmol} \cdot \mathrm{mg}^{-1}$). Eqn. 1 is then substituted into the following three equations:

$$v = (\mathrm{SA})[\mathrm{E}]/B \qquad (2)$$

$$\Delta A = \Delta\epsilon([\mathrm{E}]_t - [\mathrm{E}]) \qquad (3)$$

$$F = 1 - \mathrm{Eff}(1 - [\mathrm{E}]/[\mathrm{E}]_t) \qquad (4)$$

where SA is the specific activity, and $\Delta\epsilon$ is the extinction coefficient difference. A more detailed derivation of the equation used for enzyme inhibition and an example of its use with a high-stoichiometry case can be found in Anderson et al. [19].

Kinetic and dissociation parameters were calculated with a BASIC computer program which uses a nonlinear regression algorithm [20] to fit the data. An adequate fit of the data involving quenching of FITC fluorescence, however, required a two-binding-site model. The complex analytical solution for this model was obviated by using a computer subroutine to calculate iteratively the free ligand concentration, [L], just as is done to calculate free metal and ligand concentrations from given total concentrations. The relative fluorescence was then computer fitted to:

$$F = (1 - \mathrm{Eff}\ \alpha_1)(1 - \alpha_2)$$

where α_i is $[\mathrm{L}]/([\mathrm{L}] + K_i)$, and K_1 and K_2 are the high- and low-affinity dissociation constants, respectively.

Results

Reactive Red 120 inhibition of the SR Ca^{2+}-ATPase

Reactive Red 120 was found to be a potent inhibitor of the SR Ca^{2+}-ATPase. At a given concentration of dye, the degree of inhibition was independent of the concentration of ATP (0.5–10

mM); it is therefore a noncompetitive inhibitor, binding at a site distinct from the active site. This was unexpected, because for the solubilized enzyme we observed competition between dye binding and active site occupancy [7]. Fig. 2 shows the effect of increasing concentrations of Reactive Red 120 on the velocity of ATP hydrolysis. The inhibition occurred at low concentrations of dye, indicating that the K_d of Reactive Red 120 is in the range of the total binding site concentration present in the assay. Accordingly, the I_{50} for Reactive Red 120 varies with SR concentration (Fig. 2, inset). The data were treated using Eqn. 2, which assumes simple noncompetitive inhibition and allows one to account for the depletion of ligand due to tight binding [19]. This analysis of the data yielded a value for K_i of 30 ± 10 nM and a stoichiometry of 7 ± 1.6 nmol · mg^{-1}. In this same range of dye concentrations, the calcium-independent ATPase activity showed no inhibition by Reactive Red 120.

The stoichiometry determined from inhibition

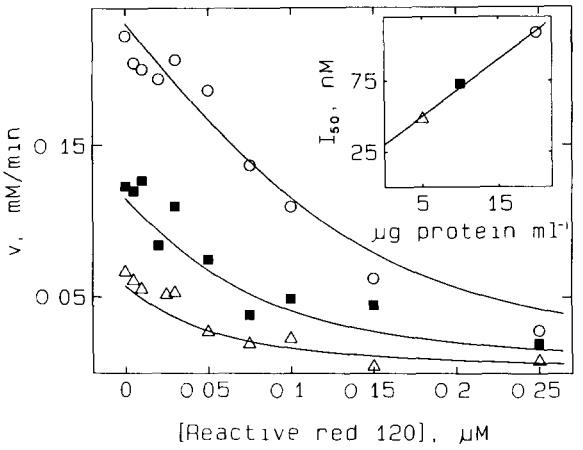

Fig. 2 Inhibition of SR vesicle hydrolysis of ATP by Reactive Red 120 SR hydrolysis of 1 mM ATP was measured by initial velocities using the phosphomolybdate assay; conditions were as described in the Materials and Methods section The Ca^{2+}-ATPase activity was measured with total Reactive Red 120 concentration varied between 0 and 0 25 μM with the following SR protein concentrations. Δ, 5, ■, 10; and ○, 20 μg/ml The lines were generated using Eqns. 1 and 2 and the fitted parameters which were obtained as described in Materials and Methods – Data analysis The fitted data yielded a value for K_d of 30 ± 10 nM and a value for the stoichiometry of 7 ± 1 6 nmol per mg SR protein. Inset· the variation of I_{50} for Reactive Red 120 inhibition as a function of SR concentration

kinetics was confirmed by direct measurements of free dye concentration. Fig. 3 shows the results of experiments in which varying concentrations of Reactive Red 120 were incubated with SR; the vesicles were then pelleted by centrifugation and the supernatants were analyzed spectrophotometrically for free dye. As seen in Fig. 3, the free dye concentration plotted as a function of total dye added showed a break occurring at about 6.8 nmol · mg^{-1} SR protein. The results shown in this figure also confirm the noncompetitive nature of the inhibition, since FITC-modified SR Ca^{2+}-ATPase (which has access by nucleotide substrates to its active site blocked) showed approximately the same stoichiometry of tight binding. The stoichiometries obtained from these experiments and those described below are all in the range of total Ca^{2+}-ATPase content of about 5 nmol · mg^{-1}, so that at these low dye concentrations we observe no significant binding of Reactive Red 120 to other SR components.

Reversibility of Reactive Red 120 binding to the Ca^{2+}-ATPase

Reversibility of inhibition was studied in order

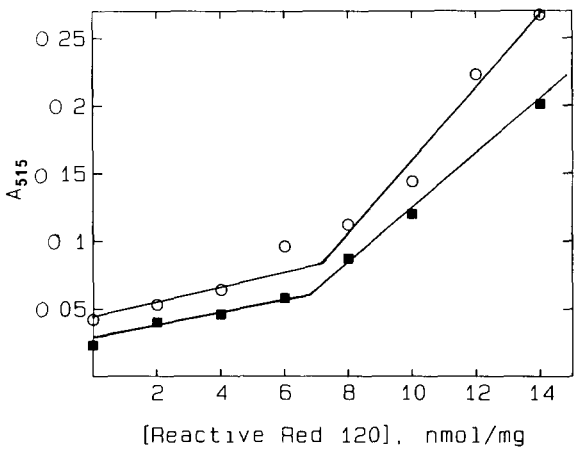

Fig 3. Binding of Reactive Red 120 to SR membranes as studied by centrifugation To 2 ml of 1 mg/ml SR vesicles in activity assay buffer was added varying concentrations of Reactive Red 120 The vesicles were then pelleted by centrifugation at 48000 × *g* for 30 min at 5 °C. The decanted supernatant's absorbance was then measured at 515 nm for unmodified SR, ■, and FITC-modified SR, ○ The breaks in the plots of free Reactive Red 120 vs. total added dye indicate stoichiometry of tight binding of approx. 7 nmol per mg SR protein for both enzymes

to determine whether the dye was covalently modifying the enzyme SR vesicles were incubated in the presence of 14 nmol of Reactive Red 120 per mg of protein. Assaying aliquots of this solution over a period of hours revealed no time-dependent loss of ATPase activity. Since bovine serum albumin binds triazine dyes tightly [25], it was added as a scavenger in some of these assays Its addition did not affect the activity of the uninhibited enzyme; when added to the dye-inhibited enzyme assay, full Ca^{2+}-ATPase activity was restored.

In preliminary experiments, we observed that Reactive Red 120 was not completely removed from SR vesicles by gel filtration on Sephadex G-50 or overnight dialysis. To investigate the possibility that covalent modification was occurring at sites which do not affect ATP hydrolysis, samples of SR which had been incubated under the above conditions were analyzed by SDS gel electrophoresis. The samples were run on 12% tube gels according to the method of Laemmli [10]. Subsequent scans at 525 nm revealed that no dye was present in the area of the M_r 100000 Ca^{2+}-ATPase (the migration of the Ca^{2+}-ATPase was determined from control runs using the FITC-modified enzyme). In these electrophoresis experiments, Reactive Red 120 ran as a single band with an R_F of 0.8 relative to the tracking dye, Bromphenol blue. Reactive Red 120 (in the absence of protein) migrated with the same R_F. Gels which were fixed and subsequently destained showed no remaining Reactive Red 120. The above results indicate that under these conditions (Reactive Red 120 present at concentrations saturating the tight binding sites and overnight incubation at room temperature), no covalent modification of SR proteins occurs.

Ca^{2+}-ATPase inhibition by related dyes

Since Reactive Red 120 posseses two chromophoric domains per molecule, the inhibitory potency of related but smaller dyes (Fig. 1) toward the Ca^{2+}-ATPase was studied. Reactive Red 8, which is structurally similar to one-half of the Reactive Red 120 molecule, also exhibited a degree of inhibition that was independent of [ATP]. Thus it is also a noncompetitive inhibitor, and its K_i was determined to be 8.8 ± 2.6 μM, indicating it binds almost 300-fold more weakly than Reactive Red 120 does. A somewhat larger triazine dye, Reactive blue 2, has, when immobilized on agarose, been used to purify the solubilized SR Ca^{2+}-ATPase [26]; after ascertaining that it also acted as a noncompetitive inhibitor, we found its K_i to be 0.81 ± 0.13 μM. Clearly, the structure of Reactive Red 120 enables it to bind far more tightly (over 25-fold) than the smaller dyes. When dye binding was analyzed by the centrifugation method illustrated in Fig. 3, we could find no evidence for competition between Reactive Red 120 and Reactive Blue 2. This was also the case when the spectral absorption of the SR–Reactive-Red-120 complex was monitored in the presence of Reactive Blue 2 (data not shown). These results imply that Reactive Red 120 binds to a unique site having little affinity for other triazine dyes.

Effect of nonionic detergents on the concentration of free dyes

Part of this study is concerned with the interaction of triazine dyes with the detergent-solubilized Ca^{2+}-ATPase as compared with their interaction with the membranous enzyme. We have therefore studied the behavior of Reactive Red 120 and Reactive blue 2 in solutions of non-ionic detergent and the effect of detergent on dye inhibition of the soluble enzyme, lactate dehydrogenase. The critical micelle concentration (cmc) for the non-ionic detergent, $C_{12}E_8$, has been determined to be 0.09 mM [21]; we have assumed the same cmc for the detergent used in this study, $C_{12}E_9$, accordingly, the micelle concentration for the present work was taken as proportional to the total detergent concentration minus the cmc. Titrations of 5 μM dye solutions (to avoid dye stacking, [22,23]) with increasing concentrations of $C_{12}E_9$ yielded the following values. Reactive Blue 2 (Fig. 1) 'bound' to the detergent micelles with CE_{50} of 0.037 mg/ml, producing a $\Delta\epsilon_{655}$ of 6170 ± 50 $M^{-1} \cdot cm^{-1}$. Reactive Red 120 (Fig. 1) bound to the detergent micelles with a CE_{50} of 0.22 mg/ml, producing a $\Delta\epsilon_{550}$ of 24800 ± 300 $M^{-1} \cdot cm^{-1}$. Accordingly, apparent decreases in enzyme affinity for these dyes due to the partitioning of the dyes into micelles should show this dependence on detergent concentration. (Assuming that the only effect of added detergent is to lower the free concentra-

tion of inhibitor, then the observed (apparent) K_i at a given concentration of $C_{12}E_9$ is: $K_{i,app} = K_i(1 + ([C_{12}E_9] - cmc)/CE_{50})$, where CE_{50} is the concentration of detergent at which half the dye is partitioned into the micelles. Accordingly, plots of $K_{i,app}$ vs. $([C_{12}E_9] - cmc)$ should be linear and the slope is equal to K_i/CE_{50}.)

Detergent–dye interactions with nonmembranous enzymes

Studying the competitive inhibtion of the soluble enzyme, lactate dehydrogenase, by Reactive Blue 2 as a function of detergent concentration, we obtained the following results. The apparent K_i obtained at different concentrations of $C_{12}E_9$ extrapolated linearly to the K_i determined in the detergent-free case; the slope of the detergent effect on apparent K_i yielded a CE_{50} of 0.048 mg/ml, in reasonable agreement with the spectrally determined value.

Lactate dehydrogenase is competitively inhibited by Reactive Red 120 also, and this strong inhibition is attenuated by detergent. The kinetic behavior is not simple, however; in the presence of Reactive Red 120, the NADH concentration dependence of the velocity is sigmoidal (data not shown). The dye, therefore, appears to be able to bridge two subunits of this enzyme. Its behavior toward pyruvate kinase is simpler Consistent with the number of nucleotide sites in each subunit [24], this enzyme bound two Reactive Red 120 molecules per subunit (Table I). Because of the complexity of the kinetic behavior displayed by lactate dehydrogenase, determination of K_i values was not attempted. Along with the binding stoichiometry of 0.5 mol per mol subunit we obtained (Table I), these results suggest that Reactive Red 120 strongly inhibits lactate dehydrogenase only when each of the two chromophores of the molecule binds to a separate active site on the tetrameric enzyme.

Effect of nonionic detergent on dye inhibitor potency toward the Ca^{2+}-ATPase

The above approach of studying the effect of detergent on the affinity of lactate dehydrogenase toward triazine dyes was extended to the Ca^{2+}-ATPase. In addition to raising of apparent K_i values by dye partitioning into the micelles, detergents may affect the affinities of ligands for membrane proteins by changing the nature of the binding site. In the present case detergents have been shown to produce active monomeric Ca^{2+}-ATPase [27–31]. Fig. 4 shows the effect of detergent concentration on the inhibition of the Ca^{2+}-ATPase by Reactive Blue 2. As can be seen, the apparent K_i extrapolates to the inhibition constant observed in the absence of detergent (SR vesicles). The slope of detergent concentration on apparent K_i yields a value for CE_{50} of 0.01 mg/ml, which is lower than that determined from spectral titrations, but is still consistent with dye partitioning as the major effect.

Fig. 5 shows the effect of detergent concentra-

TABLE I

BINDING OF TRIAZINE DYES TO SR VESICLES, LACTATE DEHYDROGENASE AND PYRUVATE KINASE

All determinations were performed at 37°C in the activity assay buffer described in Materials and Methods n d , not determined

Dye	SR vesicles [a]		Lactate dehydrogenase [b]			Pyruvate kinase [b]		
	K_i (nM)	B (nmol mg^{-1})	K_d (nM)	B (molecules subunit)	$\Delta\epsilon$ (λ,nm) (M^{-1} cm^{-1})	K_d (nM)	B (molecules subunit)	$\Delta\epsilon$ (λ,nm) ($M^{-1}\cdot cm^{-1}$)
Reactive Red 120	30 ± 10	7 ± 1 6	40 ± 30	0 49 ± 0 02	33400 ± 200 (557 5 – 500)	910 ± 100	2 0 ± 0.1	30000 ± 300 (562 – 502)
Reactive Blue 2	820 ± 100	n d	1 300 ± 1 000	1.2 ± 0 2	3500 ± 300 (660 – 575)	1000 ± 800	1 3 ± 0.2	4200 ± 400 (685 – 590)

[a] The affinities and stoichiometries were determined from the noncompetitive inhibition of ATP hydrolysis as outlined in Materials and Methods and Results and Discussion

[b] The parameters for dye binding were determined spectrally as outlined in Materials and Methods

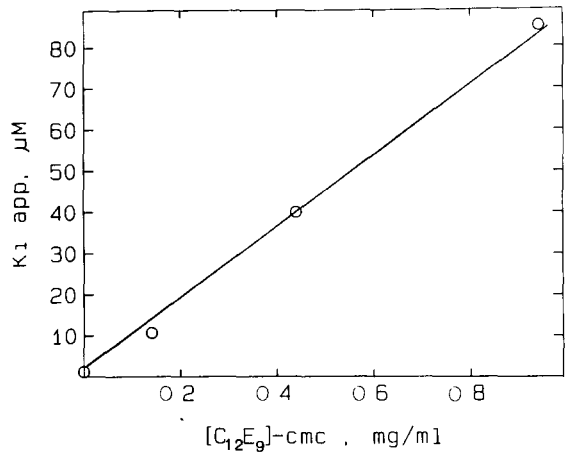

Fig 4 Inhibition of soluble Ca^{2+}-ATPase activity by Reactive Blue 2 as function of detergent concentration. Soluble SR Ca^{2+}-ATPase activity was followed using the coupled enzyme assay as described in Materials and Methods $[C_{12}E_9]-cmc$ was taken as proportional to the micelle concentration, $K_{i,app}$ is the observed K_i determined at each concentration of detergent The extrapolated K_i at zero detergent was $0\,82 \pm 0\,1$ μM, the slope of the effect of detergent on $K_{i,app}$ gave a value for the partitioning of dye into micelles of $CE_{50} = 0\,01 \pm 0\,002$ mg/ml

tion on the K_i of Reactive Red 120 toward the SR Ca^{2+}-ATPase; the value of CE_{50} calculated from the slope of the detergent-caused increase in K_i is

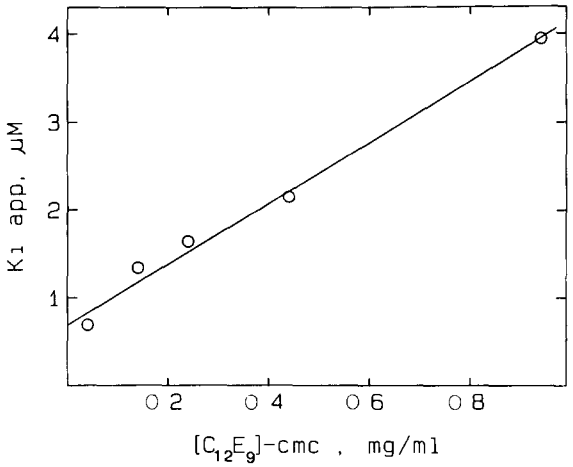

Fig 5 Inhibition of soluble Ca^{2+}-ATPase activity by Reactive Red 120 as a function of detergent concentration The conditions are as described in the legend to Fig 4 The extrapolated value of K_i at zero detergent concentration was $0\,7 \pm 0\,1$ μM, the slope of the effect of detergent on $K_{i,app}$ gave a value for the partitioning of dye into micelles of $CE_{50} = 0\,2 \pm 0\,03$ mg/ml

0.2 mg/ml, in good agreement with the absorbance-determined value. In the case of Reactive Red 120 inhibition, the extrapolated K_i at zero detergent is 700 nM, or approx. 25-fold higher than the directly observed value (SR vesicles) of 30 nM. The data clearly indicate that solubilization of the Ca^{2+}-ATPase dramatically lowers the affinity of the enzyme toward Reactive Red 120 such that the dye's K_i becomes similar to that of Reactive Blue 2. (Since experimental difficulties would not allow the use of the phosphomolybdate assay in the presence of detergents, these data were obtained using a coupled assay utilizing pyruvate kinase and lactate dehydrogenase [12]. As these two enzymes (present in high concentrations) bind triazine dyes, it was necessary to determine the free dye concentration for each case using the K_d values and stoichiometries listed in Table I. With each experimental condition, several concentrations of pyruvate kinase and lactate dehydrogenase were used; the small increases in velocity observed on doubling both enzymes ensured proper coupling and allowed the reaction rates to be related to the free dye concentration. In this manner, reliable K_i values were acquired for the dye inhibition of the soluble Ca^{2+}-ATPase.)

Effect of Reactive Red 120 on steady-state enzyme phosphorylation

Since Reactive Red 120 abolishes Ca^{2+}-ATPase activity with a low stoichiometry, it was of interest to begin to investigate which step of the catalytic cycle is inhibited by the dye. We found that Reactive Red 120 has no significant effect on the steady-state level of phosphorylated enzyme intermediate, even at concentrations of dye which reduce turnover to a few percent of normal. This result shows that Reactive Red 120 does not solely inhibit the phosphorylation step of the cycle. However, because the phosphorylation step is much faster than turnover, this result would be obtained if the dye slows down each step of the cycle by a similar degree. To investigate further the mode of inhibition, the effect of large amounts of Reactive Red 120 (20–40 nmol $\cdot$ mg^{-1}) on enzyme phosphorylation and dephosphorylation was studied. Fig. 6 shows that under these conditions no transient is observed in the formation of phosphoenzyme and no significant turnover occurs.

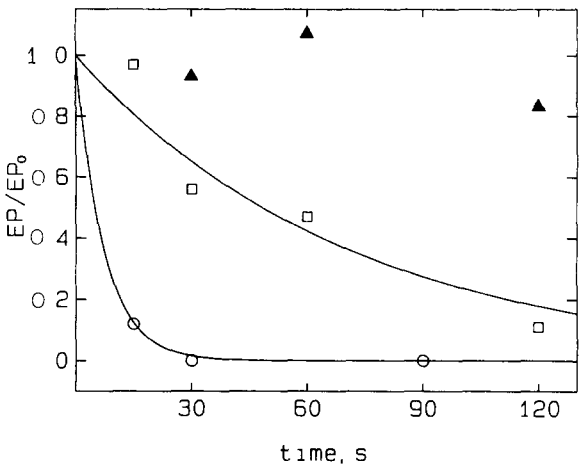

Fig 6 Effect of Reactive Red 120 on dephosphorylation of EP To a reaction mixture containing 0 2 mg ml^{-1} leaky SR vesicles and 35 μM [γ-^{32}P]ATP was added 6 mM EGTA at time zero Aliquots were quenched at the designated times and the amount of EP was determined for control SR (○), SR + 40 nmol Reactive Red 120 per mg protein (▲), SR + 40 nmol Reactive Red 120 per mg protein + 500 μM ADP added with the EGTA (□) The values of EP_0 (EP determined prior to the addition of EGTA) were 4 6 (○), 3.0 (▲) and 3 5 (□) nmol mg^{-1} Essentially the same results were obtaining at 10 nmol Reactive Red 120 per mg protein. Other details are given in Materials and Methods

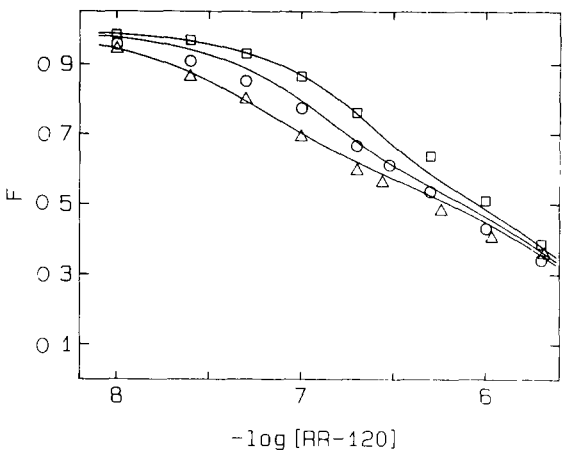

Fig 7 Effect of Reactive Red 120 on the fluorescence of FITC SR. Labeled SR (5 nmol FITC per mg protein) was titrated with Reactive Red 120 at 25 °C in 50 mM Mops/80 mM KCl/5 mM $MgCl_2$/1 mM EGTA (pH 7 0) Protein concentrations were 10 (△); 50 (○), and 100 (□) μg ml^{-1} Additional experimental details are given in Materials and Methods The lines were generated using Eqn 5 and the fitted parameters For the high-affinity binding, these are listed in Table II. The low-affinity dissociation constant obtained was 3 1 ± 0 9 μM

Addition of 500 μM ADP to the inhibited phosphoenzyme resulted in a transient decrease of EP (formation of ATP) with a $t_{\frac{1}{2}}$ of 50 s. In the absence of inhibitor, $t_{\frac{1}{2}}$ is 5 s [32].

Intersite distance measurements using FET

In order to obtain an estimate of the location of the Reactive Red site relative to the ATP catalytic site, we carried out FET experiments on SR vesicles labeled with FITC. As shown in Fig. 7, addition of Reactive Red 120 causes quenching of the fluorescence of the fluorescein chromophore. As observed for the effect of Reactive Red on Ca^{2+}-ATPase activity (Fig. 2), the degree of quenching by a given concentration of Reactive Red 120 depended on the SR concentration. From fitting the data, we estimated a binding stoichiometry of 3.2 nmol per mg, which is somewhat lower than that obtained from the inhibition results. We also obtained two dissociation constants from the results; the high-affinity value (40 nM) is in approximate agreement with the K_i obtained from the Ca^{2+}-ATPase activity measurements. Parallel experiments showed that there was no change in the absorption spectrum of FITC SR as Reactive Red 120 was added. Based on a calculated R_0 of 56 Å, the distance between the FITC and the Reactive Red sites is estimated to be 59 Å.

We also observed that the intrinsic fluorescence of SR vesicles was partially quenched in a saturable manner by Reactive Red 120. The ATPase contains 13 tryptophan residues [3], so that FET from them to the bound dye cannot be considered to be a measure of a unique distance on the protein. Nevertheless, since there is reason to be-

TABLE II

DISTANCES ESTIMATED FROM FLUORESCENCE ENERGY TRANSFER WITH REACTIVE RED 120 AS ACCEPTOR

Donor	Acceptor binding		R_0 (Å)	Efficiency	R (Å)
	B (nmol mg^{-1})	K_d (nM)			
FITC	3 2 ± 0 6	40 ± 11	56	0.43 ± 0 03	59
Trp	4 2 ± 1.3	200 ± 50	32	0 37 ± 0 02	35

lieve that most of the tryptophans are located in a membrane-spanning domain (see Discussion), estimation of an average distance has some value. The apparent K_d we obtain (0.2 μM) is somewhat higher than the ATPase inhibition and fluorescein moiety quenching counterparts; this may be related to the heterogeneity of the donor population. From the quenching efficiency at saturation and an R_0 value of 32 Å, we estimate an average distance of 35 Å. These FET results are summarized in Table II.

Effect of Reactive Red 120 on two-dimensional array formation

Because of the possibility that Reactive Red 120 may inhibit one or more conformation changes of the catalytic cycle, we tested its effect on the conformation change resulting in two-dimensional array formation [33]. When present in an amount which reduced Ca^{2+}-ATPase activity to a few percent of control (10 nmol of dye per mg of protein), we observed that Reactive Red 120 did not inhibit array formation induced by vanadate (0.5 mM decavanadate incubated with 1 mg/ml SR [34]). At these levels, the dye itself did not induce the formation of arrays; the appearance of negatively stained vesicles in electron micrographs was indistinguishable from that of vesicles without Reactive Red 120 present (Scales, D., personal communication)

Discussion

This study has shown that Reactive Red 120 binds noncovalently to SR memrbanes with high affinity and low stoichiometry. The mean ($\pm$S.D.) of the four measurements is 5.3 ± 1.9 nmol $\cdot$ mg^{-1}; this is not significantly different from our previously reported estimate of ATPase content of 4.7 nmol per mg SR protein [7]. Since the inhibition is noncompetitive and dye binding is not eliminated by FITC labeling, Reactive Red 120 binds to a part of the enzyme distinct from both the active site and the putative regulatory site. Just as the ouabain-binding site of the $(Na^+ + K^+)$-ATPase and the aurovertin site of the mitochondrial F_1-ATPase [35] have been valuable in the study of these enzymes, the Reactive Red 120 site provides the potential for further elucidation of the structure and transport cycle of the Ca^{2+}-ATPase. Thus, for example, not only does Reactive Red 120 provide a unique site on the enzyme for FET experiments, but its noncompetitive inhibition may reveal information about the steps involved in active transport.

This initial investigation as to the mode of Reactive Red 120's inhibition has shown that enzyme phosphorylation cannot be the sole affected step because the dye has at most modest effects on steady-state levels of EP. That the dye profoundly inhibits dephosphorylation of the enzyme by water or ADP suggests that Reactive Red 120 acts by stabilizing the enzyme in the Ca_2EP form (ADP-sensitive phosphoenzyme).

Since the SR Ca^{2+}-ATPase can be solubilized to produce active monomeric enzyme [27–31], we studied the interaction of triazine dyes with the enzyme in the presence of detergent. Our results show that detergent solubilization does not affect the binding affinity of Reactive Blue 2 to SR, whereas it greatly reduces the affinity of the enzyme for Reactive Red 120. The result obtained with Reactive Blue 2 is similar to those reported for high-affinity ATP and calcium binding; for both of these ligands, detergent solubilization was shown to have no signficant effect [36]. That a dramatic decrease in affinity toward Reactive Red 120 occurs upon solubilization of the SR Ca^{2+}-ATPase lends further support to the conclusion that the two triazine dyes have separate binding sites.

Previously we used Reactive Red 120 coupled to agarose to purify the detergent-solubilized SR Ca^{2+}-ATPase [7]. In this case it was found that the enzyme was eluted from the column with the substrate analogue, Ado*PP*[NH]*P*. When studying the kinetic behavior of Reactive Red 120 with the soluble Ca^{2+}-ATPase we found mixed noncompetitive kinetics (data not shown) The ability of ATP and its analogues to antagonize the binding of solubilized enzyme to Reactive Red 120 is completely different from the strict noncompetitive inhibition seen with the membranous enzyme. These results indicate that either solubilization dramatically affects the affinity and mode of inhibition by Reactive Red 120, or that upon solubilization dye binding to the active site and noncompetitive site has similar affinity. Because de-

tergent micelles bind Reactive Red 120 and because the solubilized enzyme has a much lower affinity for the dye, we were unable to determine a stoichiometry of binding with the purified Ca^{2+}-ATPase [7].

The structural features of Reactive Red 120 which allow it to bind tightly to its enzyme site (while the other dyes show little affinity) probably include its larger size and its possession of two chromophore domains. While the high-affinity binding of Reactive Red 120 may be due solely to its large number of hydrophobic and ionic interactions with a site on the Ca^{2+}-ATPase, it could also be due to one dye bridging two neighboring ATPases. This mechanism would place each of the two chromophore moieties of Reactive Red 120 in a separate dye binding site on two adjacent enzymes. Such a chelating effect would produce the observed high-affinity binding, and solubilization (monomerization) would attenuate the effect. This intriguing possibility is given credence by reports which suggest that the Ca^{2+}ATPase exists in the membrane as dimers [37,38]. The data shown in Table I suggest that Reactive Red 120 inhibits lactate dehydrogenase by such a mechanism, since only 0.5 mol dye per mol subunit is necessary for complete inhibition. The inhibition kinetics observed with Reactive Red 120 and lactate dehydrogenase, i.e., nonhyperbolic (cooperative) effects of NADH, also supports this scheme. Nevertheless, it should be noted that for the Ca^{2+}-ATPase the effect of detergent could instead be due to conformational alterations of the dye-binding site. The observed binding stoichiometry is more consistent with this interpretation.

While our results do not permit pinpointing of the location of the Reactive Red 120 site on the Ca^{2+}-ATPase, they provide some information about the structure of the enzyme. With the publication of the complete amino-acid sequence, MacLennan et al. [3] postulated a model which involves phosphorylation and nucleotide-binding domains connected by a calcium-binding stalk to a transmembrane segment. Examination of this model reveals that 11 of the enzyme's 13 tryptophan residues would be located in this transmembrane domain. Assuming the nearest of these residues are close to the center of a 60-Å-thick bilayer, our results would place the Reactive Red 120 site in the stalk region near the phospholipid head groups (Fig. 8).

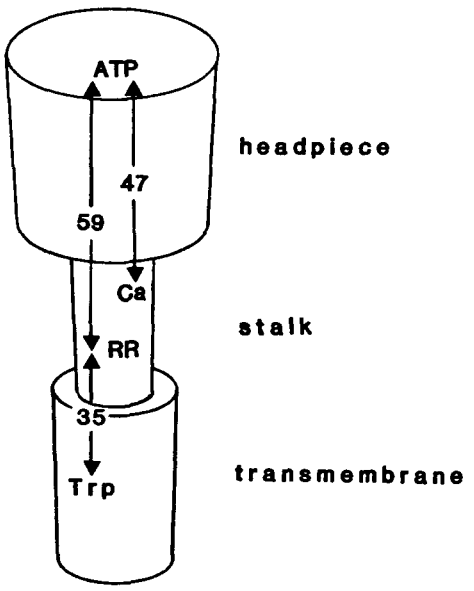

Fig 8. Structural model of the Ca^{2+}-ATPase The volumes of the three cylinders are proportional to the proposed number of amino-acid residues in the transmembrane, stalk, and nucleotide-phosphorylation (headpiece) domains [3] The height of each cylinder is taken to be 40 Å For the transmembrane and stalk regions, this is based on hypothetical six-turn helices [3] For the headpiece this height is arbitrary. The diameters of the cylinders are calculated from the molecular volumes and the heights. Intersite distances. Trp–RR (donor: tryptophan sidechains, acceptor, Reactive Red 120), ATP–RR (donor FITC, acceptor Reactive Red 120), Ca–ATP (donor· Tb, acceptor FITC [39]. The colinearity of Trp–RR–ATP is arbitrary

At a distance of about 60 Å from the Reactive Red 120 site, the catalytic site according to our results would be located in a different domain, as is predicted by the sequence model. Placing the nucleotide site at some distance from the calcium-binding sites is also consistent with the results of recent FET studies involving this site (Fig. 8) [9,39]. Given the postulated small size (about 100 residues according to MacLennan et al. [3]) of the stalk domain, its containing both the calcium sites and a dye site would place them rather near one another.

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References

1 De Meis, L. (1981) The Sarcoplasmic Reticulum Transport and Energy Transduction, Wiley, New York
2 Inesi, G (1985) Annu Rev Physiol 47, 573–601
3 MacLennan, D H, Brandl, C J, Corczak, B and Green, N M (1985) Nature (Lond) 316, 696–700
4 Verschoor, G J, Van der Sluis, P R and Slater, E C (1977) Biochim Biophys Acta 462, 438–449
5 Satre, M Bob, M and Vignais, P V (1980) J Bacteriol 142, 768–776
6 McEnery, M W and Pedersen, P L (1986) J. Biol Chem 261, 1745–1752
7 Coll, R J and Murphy, A J. (1984) J Biol Chem 259, 14249–14254
8 Eletr, S and Inesi, G (1972) Biochim Biphys Acta 282, 174–179
9 Highsmith, S and Murphy, A.J (1984) J Biol Chem 259, 14651–14656
10 Laemmli, U K (1970) Nature (Lond) 227, 680–685
11 Coll, R J and Murphy, A J (1985) FEBS Lett 187, 131–134
12 Anderson, K W and Murphy, A J. (1983) J Biol Chem 258, 14276–14278
13 Fromm, H J (1963) J Biol Chem 238, 2938–2944
14 Bucher, T and Pfeiderer, G (1955) Methods Enzymol 1, 435–440
15 Thompson, S T and Stellwagen, E (1976) Proc Natl Acad Sci USA 73, 361–365
16 Fairclough, R H and Cantor, C R (1978) Methods Enzymol. 48, 347–379
17 Chen, R F (1967) Anal Lett 1, 35–42
18 Cantley, L C and Hammes, G G (1975) Biochemistry 14, 2976–2981
19 Anderson, K W, Coll, R.J and Murphy, A J (1984) J Biol Chem 259, 11487–11490
20 Bevington, P R (1969) Data Reduction and Error Analysis for the Physical Sciences, McGraw-Hill, New York
21 Tanford, C (1980) The Hydrophobic Effect, Formation of Micelles and Biological Membranes, 2nd Edn, p 66, Wiley, New York
22 Edwards, R A and Woody, R W (1979) Biochemistry 18, 5197–5204
23 Federici, M M., Chock, P B and Stadtman, E.R. (1985) Biochemistry 24, 647–660
24 DeCamp, D L. and Colman, R F (1986) J Biol Chem 261, 4449–4503
25 Ticha, M, Horejsi, V. and Barthova, J (1978) Biochim Biophys Acta 534, 58
26 Gafni, C and Boyer, P D (1984) Biochemistry 23, 4362–4367
27 Dean, W L and Tanford, C (1978) Biochemistry 17, 1683–1690
28 Møller, J.V, Lind, K E and Andersen, J P (1980) J Biol Chem 255, 1912–1920
29 Murphy, A J, Pepitone, M and Highsmith, S (1982) J Biol Chem 257, 3551–3554
30 Klemens, M R, Andersen, J P and Grisham, C M (1986) J Biol Chem 261, 1495–1498
31 Vilsen, B and Andersen, J.P (1986) Biochim Biphys Acta 855, 429–431
32 Shigekawa, M and Dougherty, J P (1978) J Biol Chem 253, 1451–1457
33 Dux, L and Martonosi, A (1983) J Biol Chem 258, 11896–11902
34 Coan, C, Scales, D J. and Murphy, A J (1986) J Biol Chem 261, 10394–10403
35 Lunardi, J, Klein, G and Vignais, P V (1986) J Biol Chem. 261, 5350–5354
36 Kosk-Kosicka, D, Kurzmack, M and Inesi, G. (1983) Biochemistry 22, 2259–2267
37 Scales, D and Inesi, G. (1976) Biophys J 16, 735–751
38 Hymel, L., Maurer, A, Berenski, C, Jung, C Y and Fleischer, S (1984) J Biol Chem 259, 4890–4895
39 Scott, T L (1985) J Biol. Chem 260, 14421–14423