PROBING THE NUCLEOTIDE BINDING SITES OF SARCOPLASMIC RETICULUM ATPASE BY PHOTOAFFINITY LABELING

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The Ca-ATPase of sarcoplasmic reticulum is a membrane-bound enzyme consisting of a single polypeptide chain of molecular weight 100,000–120,000 (1). The partial reactions of the catalytic and transport cycles of the enzyme have been extensively studied (2). However, the functional unit of the ATPase has not yet been clearly understood (1). The enzyme displays a nonMichaelian behavior for the ATPase activity with respect to Mg ATP, which has been attributed to an effect of ATP at low affinity regulatory sites (3–6). Alternatively, a half-of-the-sites reactivity mechanism has been suggested to explain the modulation of the turn-over rate by ATP (7, 8) and a possible interaction of subunits has been implicated (9, 10). As the stoichiometry of nucleotide binding sites of the enzyme is still controversial, it is difficult at present to decide which is the most likely mechanism.

Photoaffinity labeling is an advantageous technique to clarify the stoichiometry and/or the topography of ATP binding sites (11), as it allows covalent attachment of nucleotides at possible regulatory sites. In the sarcoplasmic reticulum ATPase the putative regulatory sites cannot be titrated by conventional binding techniques because of their low affinity (12). Recently, Cable and Briggs (13) reported the photoaffinity labeling of a small fraction of the Ca-ATPase high-affinity catalytic sites by benzoyl-benzoic ATP. We have been involved in the characterization of high and low affinity classes of nucleotide binding sites using fluorescent (14) and photoactivatable (15) ATP analogs. The fluorescent analog 3′-trinitrophenyl-ATP (TNP-ATP) revealed the existence of two classes of sites (14) but the fluorescence intensities measured did not permit the evaluation of the stoichiometry. In addition, TNP-ATP is not a substrate for the Ca-ATPase in that it is not hydrolyzed by the enzyme (14).

To investigate the stoichiometry of the catalytic and regulatory nucleotide sites in the Ca-ATPase we have chosen to use ATP analogs which are photactivatable and could covalently attach to the enzyme upon illumination. Both UTP and 3′-arylazido-ATP were used.

The irradiation of a mixture containing ATPase and 1 mM [^{3}H] UTP resulted in a time-dependent incorporation of UTP to the protein. The presence of nonradioactive ATP in the irradiation mixture prevented the photoincorporation of radioactive UTP; competition experiments performed with the ATP analogue TNP-ATP displayed similar protection.

To verify if ATP could be covalently attached to the ATPase we performed a series of experiments in which various concentrations of radioactive ATP were illuminated together with the enzyme. However, in our experimental conditions, we could not achieve the covalent linkage of [^{3}H]ATP to the ATPase, even when concentrations of ATP as high as 1 mM or 10 mM were used or with irradiation times as long as 2 h.

To evaluate the range of UTP concentrations that promote catalysis we measured the K_m for hydrolysis of UTP by the ATPase in the dark. The results showed that UTP has a rather low affinity for the enzyme and displays a Michaelian kinetics with a K_m of 2–3 mM. We then irradiated the ATPase in the presence of various UTP concentrations in the range of those used for the measurement of the K_m. The photolabeling displayed a hyperbolic dependence on UTP concentration very similar to that required for catalysis with an observed $K_{0.5}$ for photolabeling of ~3 mM, which was identical with the K_m for UTP hydrolysis. The fact that the extent of UTP attachment to the enzyme was saturable with a hyperbolic dependence on concentration strongly suggests that the labeling occurs exclusively at a single binding site, i.e., the catalytic site in the ATPase.

The utilization of a saturating concentration of UTP and the irradiation of the mixture for a long period of time have permitted the measurement of the maximum level of labeling. The maximum level of ~3.5 nmol [^{3}H] UTP/mg of protein is the same as the maximum level of phosphoenzyme intermediate (E-P) in the steady-state (~3.5–4 nmol E-P/mg of protein in our preparation). Therefore, taking the steady-state level of phosphoenzyme as a measure of the number of functioning units of ATPase, it can be seen that all the sites capable of being phosphorylated, i.e., the catalytic sites, are labeled by UTP. The level of photolabeling by UTP (and of phosphoenzyme accumulation) corresponds to 0.5 mol of sites/mol ATPase.

In contrast, photolabeling of the enzyme with the photoactivatable analog 3′-arylazido-ATP (aATP) indicates the existence of 1 mol of high-affinity sites and 1–1.5 mol of low-affinity nucleotide sites per mole of sarcoplasmic reticulum ATPase. The analog aATP was hydrolyzed by the enzyme in the dark. A biphasic curve of velocity of hydrolysis of the analog vs. aATP concentration was obtained, indicating the presence of high- and low-affinity sites with $K_{0.5}$ of ~10 μM and 300 μM. After irradiation

BIOPHYS. J. © Biophysical Society · 0006–3495/86/01/108/02 $1.00
Volume 49 January 1986 108–109

with visible light a biphasic curve was obtained for the level of covalent photolabeling of the enzyme vs. [β^{32}P] aATP concentrations, which was similar to the biphasic curve for the velocity of hydrolysis of the analog. Levels of 6.5–9 nmol analog/mg protein and 20–22 nmol analog/mg protein were obtained when photolabeling with 20–30 or with 400 μM aATP.

The levels obtained with aATP when photolabeling the catalytic site are twice that obtained by photolabeling with UTP and twice the steady-state level of phosphoenzyme measured. The stoichiometry of photolabel incorporated is 1 mol arylazido-ATP/mol ATPase at the catalytic site. It has been recently suggested that the stoichiometry of 0.5 mol of phosphoenzyme/mol ATPase commonly measured is related to the existence of nonfunctional inactive ATPase in the preparations (16, 17). Our results indicate that photolabeling with UTP discriminates the functional sites, whereas aATP does label all available nucleotide sites. The stoichiometry of 1 mol aATP photoincorporated/mol ATPase with a $K_{0.5}$ of ~5 μM aATP, suggests that the modification in the ATPase that leads to inactivation is not an extensive one, in that it does not eliminate the binding and photoincorporation of the nucleotide analogue.

The photoincorporation of an additional 1–1.5 mol of aATP/mol enzyme to give a final level of 2–2.5 mol of photolabel/mol of enzyme at an aATP concentration range (0.05–1 mM), which produces turnover activation in the dark, is strong evidence that the regulatory effects of the nucleotide on the turnover are related to a site on the Ca-ATPase distinct from the catalytic.

The identification of proteolytic fragments of labeled ATPase and mapping of the nucleotide binding regions of both sites are the present subject of our work and should help in understanding the relationship between structure and function in this enzyme.

This research was supported by grants from Conselho Nacional de Desenvolviments Cientifico e Tecnologico/Financiadora de Estudos e Projetos (Brazil) and the Muscular Dystropy Association (USA).

Received for publication 3 May 1985.

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