Ca^{2+} DEPENDENT EFFECT OF ACETYLPHOSPHATE ON SPIN-LABELED SARCOPLASMIC RETICULUM

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SUMMARY: Acetylphosphate produces a definite change in the spectrum of an iodoacetamide spin probe covalently bound to sarcoplasmic reticulum ATPase. The observed change, which is Ca^{2+} dependent and reversible, is attributed to a protein conformational change occurring during the Ca^{2+} transport cycle.

Vesicular fragments of SR membrane provide an isolated system displaying a highly specific Ca^{2+} pump (1-3). The exergonic reaction coupled to the pump is catalyzed by an enzyme which accounts for approximately 80% of the membrane protein (4,5). While ATP is the most specific substrate for this reaction, AcP can be utilized also (6,7). In both cases the reaction mechanism includes Ca^{2+} dependent phosphorylation of the enzyme and subsequent hydrolytic cleavage of orthophosphate (7-9). As compared to ATP, utilization of AcP requires higher Ca^{2+} concentrations and proceeds at much lower rates (10).

A very interesting question related to cation transport is whether any step of the reaction mechanism includes a protein conformational change. In this regard, we previously observed that addition of ATP to SR labeled with an iodoacetamide spin probe produces a marked spectral change (11,12). However, due to experimental limitations related to the use of ATP, we were unable

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Abbreviations: SR, sarcoplasmic reticulum; AcP, acetylphosphate; ESR, electron spin resonance; EDTA, ethylenediamine tetraacetic acid; EGTA, ethyleneglycol-bis-(B-aminoethyl ether) N,N'-tetraacetic acid; MOPS, morpholinopropane sulfonic acid.

to demonstrate an actual involvement of this effect in the transport cycle. We find now that AcP produces a spectral change similar to that obtained with ATP. It can be shown that this effect is Ca^{2+} dependent and reversible, displaying a time course parallel to that of AcP hydrolysis.

MATERIALS AND METHODS: SR vesicles were obtained from white muscle of rabbit hind legs as previously described (13).

The spin label N-(1-oxyl-2,2,6,6-tetramethyl-4 piperidyl)-iodoacetamide was purchased from SYNVAR. Labeling was obtained by incubating (2 hours at 25°C) 15 mg SR protein with 1-2 mg of the iodoacetamide label in 20 mM MOPS, 80 mM KCl, pH 6.8 (final vol: 5.0 ml). Following incubation, the unbound label was removed by repeated centrifugations and suspensions of labeled SR. Under these conditions only 2-3 SH moles/10^5g SR protein are labeled, and the ATPase and Ca^{2+} transport activities are not affected (12,14). The final suspensions to be used for ESR spectroscopy contained 13-17 mg SR protein/ml.

AcPase activity was monitored by measuring residual AcP (15) at time intervals and in conditions comparable to those used for ESR spectroscopy.

RESULTS AND DISCUSSION: The ESR spectra obtained with labeled SR are similar, but not identical, to those of other spin-labeled proteins (11,16, and 17). In Fig. 1 a "more mobile" and a "more constrained" spectral component are partially resolved and identified as "A" and "B", respectively. The two spectral components are attributed to the effects of different local environments on labels residing in two distinct site classes. The ratio between the two components may be changed by varying the extent of labeling.

In the presence of Mg^{2+}($\sim 10^{-3}$M) and Ca^{2+}($\sim 10^{-5}$-10^{-3}M), addition of AcP (10^{-3}M) to labeled SR results in a definite spectral change (Fig. 1) which appears as a decrease in the height of peak "B" as compared to that of peak "A". This effect is related to a broadening of the "more constrained" spectral component, as demonstrated by a relatively increased lineheight at an arbitrarily chosen point "C" of the field, at which point the signal intensity correspond-

ing to the "A" spectral component is expected to be minimal The broadening of the "more constrained" component is also revealed by flattening and upfield shift of minimum "D" (Fig. 1 and Table 1).

The above described spectral change is produced by AcP in mM concentration range and is reversible. In fact a reassuring

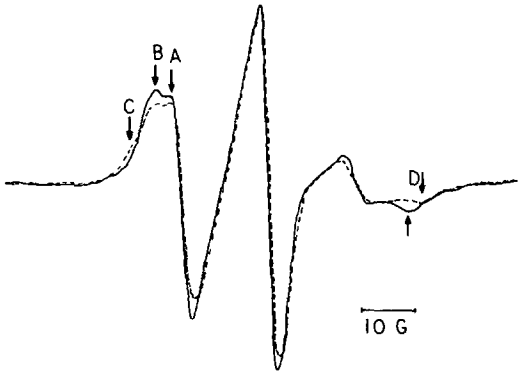

Fig. 1 ESR spectra of iodoacetamide labeled SR (15 mg protein/ml). Solid line: control (20 mM MOPS pH 6.8 and 80 mM KCl); dashed lines: after the addition of 20 mM AcP, 15 mM $MgCl_2$ and 10 mM $CaCl_2$. Spectra were obtained with a JEOL-ME 1X spectrometer at 25°C, over a 100 G range/10 minutes sweep, using a 2 G modulation width and a 3 seconds response time.

Table 1: Effects of AcP, Ca^{2+} and Mg^{2+} on ESR spectra of iodoacetamide spin-labeled SR. The ratios of lineheights at points C and A, and the splitting between peak B and minimum D (Fig. 1) are given here to provide a semiquantitative evaluation of the observed changes.

Additions	C/A	B⟷G
Control (20 mM MOPS, 80 mM KCl)	0.27	48.0 G
10 mM $CaCl_2$, 10 mM $MgCl_2$	0.27	48.0 G
20 mM AcP, 10 mM EDTA	0.29	48.5 G
20 mM AcP, 10 mM EGTA	0.28	48.5 G
20 mM AcP, 10 mM EGTA, 15 mM $MgCl_2$	0.30	49.0 G
20 mM AcP, 10 mM Ca·EGTA, 15 mM $MgCl_2$	0.39	50.0 G
20 mM AcP, 10 mM Ca·EGTA, 10 mM $CaCl_2$, 15 mM $MgCl_2$	0.38	50.0 G

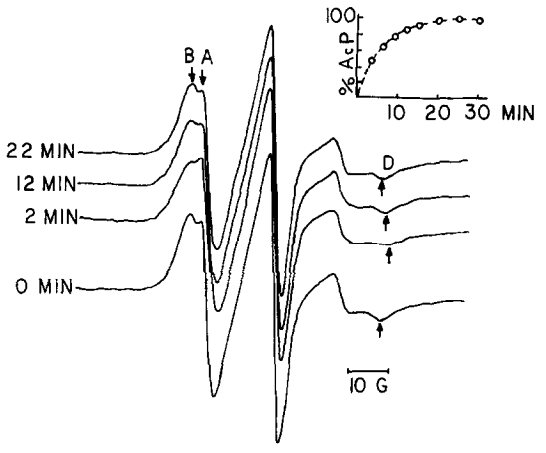

Fig. 2 Sequential scanning of the iodoacetamide labeled SR after the addition of AcP, $MgCl_2$ and $CaCl_2$. Note reversal of spectral changes in parallel with hydrolysis of the AcP (insert).

feature of the observed effect is that the ESR spectra return to their original shape in parallel with hydrolysis of the AcP added to the reaction mixture (Fig. 2).

In the absence of AcP, addition of Mg^{2+} and/or Ca^{2+} does not produce obvious spectral changes at 25°C. On the other hand, the effect of AcP is significantly less pronounced in the presence of chelating agents (10 mM EDTA or EGTA) and no added Mg^{2+} or Ca^{2+}.

A quantitative evaluation of the observed effects is rendered difficult by extensive overlapping of the two spectral components "A" and "B". However, semiquantitative estimates may be obtained by comparing lineheights ratios at arbitrary points in the field, or by measuring the splitting of the outer peaks "B" and "D". It is apparent (Table 1) that the effect of AcP on these spectral parameters is Ca^{2+} dependent. The partial effect observed in the presence of EGTA or EDTA is attributed to incomplete chelation of endogenous Ca^{2+}, as suggested by a low AcPase activity remaining in these experimental conditions. It is also important to notice

that the AcP induced spectral change is fully developed in the presence of a Ca·EGTA buffer system permitting a free Ca^{2+} concentration as low as 10^{-5}M.

The spectral features of a bound spin label, as compared to those of a free label in solution, reflect most commonly the motional constraint imposed by the local environment on the label with respect to the labeled macromolecule. The occurrence of alterations in the spectra of bound labels may be attributed to conformational changes involving a segment of the host molecule in close proximity to the label. As we have previously shown that the iodoacetamide label is in fact bound to the ATPase protein of SR (12), we attribute the spectral changes produced by AcP to a conformational change undergone by the SR ATPase.

Transport phenomena such as translocation and cyclic changes in enzyme affinity for Ca^{2+} could be explained with protein conformational effects. Evidence for such effects has been sought for a long time. In our laboratory we first observed alterations induced by ATP on the spectra of the iodoacetamide spin label bound to SR (11,12). However, due to a very high affinity of the transport system for Ca^{2+} in the presence of ATP and to a further effect of the reaction product (ADP), we were unable to demonstrate a direct relationship between the observed alterations and the transport mechanism. Subsequently, spectral changes exhibited by an N-ethylmaleimide spin label bound to SR were related to the occurrence of conformational changes during the transport cycle (18-20). The reported changes, however, were of very low intensity. Furthermore, labeling of SR with N-ethylmaleimide is carried out at alkaline pH and can cause enzyme inactivation (21). In fact, we find that labeling of -SH residues requires caution in order to avoid non specific oxidation, in addition to spin labeling.

With the experiments described in this report we have demonstrated a definite spectral alteration exhibited by spin labeled SR on addition of AcP and Ca^{2+}. The spectrum returns to its initial shape as soon as the added AcP is hydrolyzed by the labeled SR, which retains full enzyme and transport activities. The observed spectral change is similar to that previously obtained with ATP (11). AcP and ATP do not present steric similarities, but both function as substrates for the exergonic reaction coupled to Ca^{2+} transport. We suggest that the effects of these substrates on the spectra of spin labeled SR reflect the occurrence of protein conformational changes which are related to the transport cycle.

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REFERENCES

1. Hasselbach, W. and Makinose, M. (1961) Biochem. Z. 333, 518-528.
2. Ebashi, S. and Lipman, F. (1962) J. Cell Biol. 14, 389-400.
3. Hasselbach, W. and Makinose, M. (1963) Biochem Z. 339, 94-111.
4. Meissner, G. (1975) Biochim. Biophys Acta 389, 51-68.
5. Malan, N., Sabbadini, R., Scales, D. and Inesi, G. (1975) FEBS Letters 60, 122-125.
6. de Meis, L. (1969) J. Biol. Chem. 244, 3735-3739.
7. Pucell, A. and Martonosi, A. (1970) J. Biol. Chem. 246, 3389-3397.
8. Yamamoto, T. and Tonomura, Y. (1967) J. Biochem. 62, 558-575.
9. Makinose, M. (1969) Europ. J. Biochem. 10, 74-82.
10. Friedman, Z. and Makinose, M. (1970) FEBS Letters 11, 69-72.
11. Landgraf, W.C. and Inesi, G. (1969) Arch. Biochem. Biophys. 130, 111-118.
12. Inesi, G. and Landgraf, W.C. (1970) Bioenergetics 1, 355-365.
13. Eletr, S. and Inesi, G. (1972) Biochim. Biophys. Acta 282, 174-179.
14. Champeil, P., Bastide, F., Taupin, C., Gary-Bobo, C.M. (1976) FEBS Letters 63, 270-272.
15. Lipman, F. and Tuttle, L. (1945) J. Biol. Chem. 159, 21-28.
16. McConnell, H.M., Deal, W. and Ogata, R.T. (1969) Biochem. 8, 2580-2585.
17. McConnell, H.M. and McFarland, B.G. (1970) Quart. Rev. Biophys. 3, 91-136.
18. Nakamura, H., Hori, H. and Mitsui, T. (1972) J. Biochem. 72, 635. 646.
19. Pang, D.C., Briggs, F.N. and Rogowski, R.S. (1974) Arch. Biochem. Biophys 164, 332-340.
20. Tonomura, Y. and Morales, M. (1974) Proc. Nat. Acad. U.S. 71, 3687-3691.
21. Yoshida, H. and Tonomura, Y. (1976) J. Biochem. 79, 649-654.