molecular basis of variegate porphyria and to examine the possible role of this enzyme during erythropoietic differentiation.

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Phosphorylation of the Sarcoplasmic Calcium-Activated Adenosinetriphosphatase As Studied by ^{31}P Nuclear Magnetic Resonance

Gerhard M. Sontheimer,[‡] Hans Robert Kalbitzer,*[,§] and Wilhelm Hasselbach[∥]

Departments of Molecular Physics, Biophysics, and Physiology, Max Planck Institute for Medical Research, D-6900 Heidelberg, Federal Republic of Germany

Received November 7, 1986; Revised Manuscript Received January 6, 1987

ABSTRACT: A reinvestigation of a study of Fossel et al. [Fossel, E. T., Post, R. L., O'Hara, D. S., & Smith, T. W. (1981) *Biochemistry 20*, 7215–7219] in which the ^{31}P nuclear magnetic resonance (NMR) signal of the phosphointermediate of the sarcoplasmic (Ca^{2+},Mg^{2+})-ATPase has been identified shows that the signal they describe most probably originates from free Mg·ATP but not from the phosphoenzyme itself. It was possible to detect the ^{31}P NMR signal of the phosphoenzyme in peptic fragments of sarcoplasmic ATPase phosphorylated either by ATP or by inorganic phosphate. The two products exhibit the same spectral characteristics in ^{31}P NMR, implying that most probably both reaction pathways yield the same chemical product. Chemical shifts at low pH (−6.5 ppm) and high pH (−1.4 ppm) of the phosphoryl group are indicative of a β-phosphoaspartyl moiety, thus confirming independently the results from chemical analysis. The relatively low pK value of 4.3 of the phosphoryl group suggests an interaction with a positively charged group of the enzyme.

The amino acid sequence of Ca^{2+}-activated ATPase from rabbit muscle sarcoplasmic reticulum and the amino acid sequence of sheep kidney (Na^+,K^+)-ATPase have been deduced previously from their complementary DNA sequence (MacLennan et al., 1985; Shull et al., 1985). Both enzymes show significant homologies in their amino acid sequences and are probably the most intensively studied model systems for active ion-transport processes. During enzymatic catalysis, the pump protein is phosphorylated by its substrate ATP. The phosphoryl group is probably covalently bound to an aspartyl residue as Post and Kume (1973) and Bastide et al. (1973) could show by phosphorylation of the enzyme with radioactively labeled ATP and subsequent digestion of the phosphorylated protein. The active-site phosphopeptides could be isolated; they contain the sequence-Cys-Ser-Asp-Lys-Thr- (Bastide et al., 1973; Allen & Green, 1976).

A more direct approach for the study of the activity of enzymes during catalysis is the detection of phosphoenzyme intermediates by ^{31}P nuclear magnetic resonance (NMR).[1] However, the observation of phosphorylated intermediates of (Ca^{2+},Mg^{2+})-ATPase should be rather difficult due to the low concentrations obtainable. Moreover, the sarcoplasmic Ca^{2+} pump is embedded in the phospholipid matrix of the mem-

[‡] Department of Molecular Physics, Max Planck Institute for Medical Research.

[§] Department of Biophysics, Max Planck Institute for Medical Research.

[∥] Department of Physiology, Max Planck Institute for Medical Research.

[1] Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; TCA, trichloroacetic acid; NMR, nuclear magnetic resonance; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide.

0006-2960/87/0426-2701$01.50/0

brane, and if the local mobility of the phosphorus group at the active center is not high enough, the phosphorus NMR signal should be too broad to be detectable.

In spite of these difficulties, Fossel et al. (1981) reported that they had succeeded to observe the ^{31}P NMR signals of the phosphointermediates of the rabbit sarcoplasmic (Ca^{2+},-Mg^{2+})-ATPase and the (Na^+,K^+)-ATPase from the salt gland of the duck. The chemical shifts of these resonances, 17.4 and 17.5 ppm, respectively, were quite unusual for acyl phosphates. The resonance disappeared after addition of hydroxylamine, a reagent assumed to split acyl phosphate bonds. Phosphorylation of aspartic acid and of the dipeptide Ser-Asp gave signals at 11.6 and 17.4 ppm, respectively, which were assigned to the β-phosphoaspartic moiety. At least the chemical shift of the phosphorus resonance in the dipeptide was in excellent agreement with the values found for the protein.

All evidence taken together, the ^{31}P NMR signal of the phosphointermediate seemed to be well characterized. In this paper, we will report the results we obtained from this basis; as it turned out, a detailed reinvestigation of the above-mentioned data was necessary.

Materials and Methods

Materials. $MgCl_2$, $CaCl_2$, and KCl were obtained from Merck (Darmstadt); ATP was from Pharma Waldhof (Düsseldorf); EGTA, imidazole hydrochloride, Tris, and TCA were from Roth (Karlsruhe); pronase, pepsin, L-aspartic acid, L-aspartyl α-benzyl ester, L-aspartyl β-benzyl ester, glycylglycylaspartylalanine, and EDC (1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide) were from Serva (Heidelberg). All reagents were analytical grade. ATP was passed over a Chelex-100 column in order to remove divalent ions, lyophilized, redissolved in H_2O, and stored at 246 K.

The model compounds aspartic acid, aspartyl α-benzyl ester, aspartyl β-benzyl ester, and Gly-Gly-Asp-Ala were phosphorylated by reacting them in 30 mM phosphate buffer, pH 7.0 and 3.4, respectively, and 40 mM EDC. The reaction was followed by ^{31}P NMR spectroscopy of the reaction mixture at 308 K.

Sarcoplasmic reticulum was prepared from rabbit white skeletal muscle according to Hasselbach and Makinose (1963), modified by de Meis and Hasselbach (1971). The final pellet was resuspended in an appropriate volume of 50 mM imidazole hydrochloride buffer, pH 6.8, or 10 mM histidine/HCl, pH 6.0, for obtaining a protein concentration in the range of 30–45 mg/mL and dialyzed against a large volume (more than 100 times the sample volume) of the same buffer for 16–24 h. The material was used immediately after preparation. The calcium-activated ATPase activity was assayed as described by Hasselbach and Makinose (1963). With a protein concentration of 0.1 mg/mL the specific activity usually obtained was 1–1.2 μmol of P_i (mg of protein)$^{-1}$ min^{-1} at 298 K.

Phosphorylation of the Sarcoplasmic ATPase. For a reinvestigation of the work of Fossel et al. (1981), the phosphorylation was performed under the conditions described in their publication.

For the ^{31}P NMR study of the fragments obtained by digestion of phosphorylated sarcoplasmic ATPase with Pronase, the procedure described by Bastide et al. (1973) has been used.

The sample for phosphorylation with ATP and digestion with pepsin was prepared from an assay containing 5 mM $MgCl_2$, 40 mM KCl, 0.2 mM $CaCl_2$, and 300–500 mg of protein in 500 mL of 50 mM imidazole hydrochloride buffer, pH 6.8. Phosphorylation was started by addition of ATP (0.5 mM). Denaturation was initiated after 10 s by addition of 1500 mL of 6% (w/v) trichloroacetic acid. The pellets formed after centrifugation at 4000 rpm for 20 min were resuspended in 30 mM HCl and centrifuged again for 20 min at 30000 rpm. Finally, the pellet was resuspended in 10 mL of 30 mM HCl (pH 1–2). After addition of 0.5 mL of D_2O, the sample was ready for the NMR measurements. The whole preparation procedure was performed at 278 K. A control sample was prepared in parallel by using the same procedure but either omitting ATP or omitting Ca^{2+} and adding EGTA in a concentration of 2 mM. Digestion was started in the NMR spectrometer by addition of 300 mg of pepsin to the sample. During data acquisition, the temperature was raised to 288 K for accelerating the digestion.

The sample for phosphorylation with P_i contained 10 mM $MgCl_2$, 5 mM EGTA, 300–500 mg of protein, and 2 mM P_i in 500 mL of 10 mM histidine/Tris buffer, pH 6.0. The sample was reacted at 293 K for 30 min. Denaturation was started by addition of 1500 mL of 6% (w/v) ice-cold TCA. Further procedure corresponded to the phosphorylation with ATP described above. The control sample was prepared analogously but omitting inorganic phosphate.

NMR Measurements. Most of the ^{31}P NMR spectra were recorded in a 20-mm sample tube from Wilmad (Buena, NJ) with a Bruker CXP-360 spectrometer operating at 145.8 MHz. The spectrometer was interfaced to and controlled with an Aspect 2000 computer with 80K memory and a 96 MB disc system. Usually, spectra were recorded with a spectral width of 8 kHz and 4K data points (recycling time 0.33 s). The pulse width was between 12 and 16 μs, corresponding approximately to a 45° nutation angle. The sample temperature was stabilized by a stream of dry air typically at 274 or 289 K. All spectra are referenced to an external standard of 85% phosphoric acid contained in a capillary which was immersed in the sample. The chemical shift scale was defined in such a way that its values decrease with increasing magnetic field.

Part of the pH titration studies were performed at 162.0 MHz in a 10-mm sample tube using a Bruker AM-400 spectrometer. Spectral widths were 10 kHz with 8K memory used. The pH value was varied by addition of appropriate aliquots of NaOH or HCl to the sample. pH values were measured with a combination glass electrode (Ingold, Frankfurt, FRG). The pH dependence of the chemical shift was fitted to a modified Henderson–Hasselbach equation as described, e.g., by Kalbitzer and Rösch (1981). The errors given correspond to a 95% confidence level when the t test is applied to the experimental data.

Results

Reinvestigation. Figure 1a–c shows the ^{31}P NMR spectra of sarcoplasmic vesicles in a phosphorylation assay identical with that described by Fossel et al. (1981). The first spectrum (Figure 1a) has been recorded immediately after addition of ATP. Besides the resonance of free ATP, ADP, P_i, and AMP, a resonance X at approximately −19.1 ppm can be observed (marked by an arrow). Addition of 100 mM hydroxylamine (Figure 1b) to half of the sample shown in Figure 1a leads to the disappearance of the signal X and a slight downfield shift of the β-phosphorus signal of ATP. By centrifugation of the other half of the sample which was not treated with hydroxylamine, one obtains a supernatant which is virtually free from sarcoplasmic vesicles. The corresponding ^{31}P NMR spectrum (Figure 1c) consists of essentially the same signals as the initial sample; especially signal X is still observable, although no (Ca^{2+},Mg^{2+})-ATPase is present in the solution. For examination of whether the signal X is a product of the sarcoplasmic ATPase, a sample which contained no vesicles but all other components of the phosphorylation assay has been

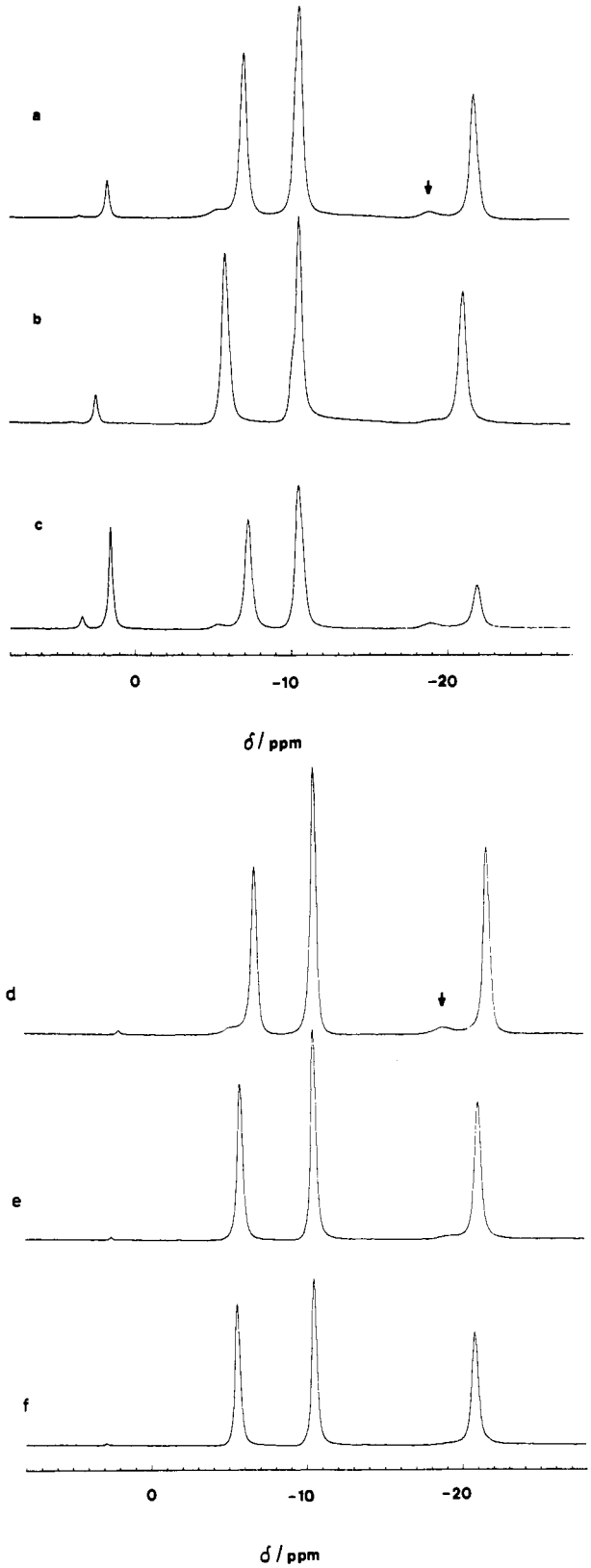

FIGURE 1: ^{31}P NMR of sarcoplasmic vesicles—control experiments. (a) Sarcoplasmic vesicles (18 mg/mL) in 50 mM imidazole hydrochloride buffer, pH 6.8, 20 μM Ca^{2+}, 0.4 mM Mg^{2+}, and 10 mM ATP. The arrow indicates resonance X. (b) Sample (a) with hydroxylamine added (100 mM). (c) Sample as in (a) centrifuged for 20 min at 30 000 rpm. Spectrum of the supernatant. Signal X is still present. (d) ^{31}P NMR spectrum of 20 μM Ca^{2+}, 0.4 mM Mg^{2+}, and 10 mM ATP in 50 mM imidazole hydrochloride buffer, pH 6.8. (e) Sample as in (d) but with hydroxylamine added to a final concentration of 100 mM. (f) Sample as in (e) but with hydroxylamine added to a final concentration of 240 mM. Line broadening of 20 Hz by exponential multiplication of the time domain data, 4000 accumulations per spectrum, temperature during the measurement 277 K.

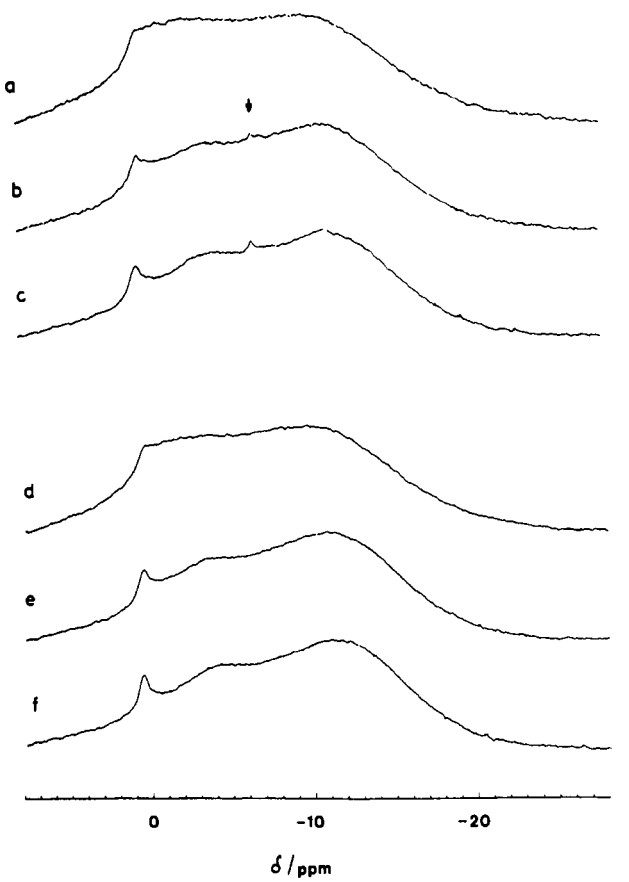

FIGURE 2: ^{31}P NMR spectra of peptic fragments of sarcoplasmic ATPase phosphorylated with ATP. (a–c) Sarcoplasmic vesicles phosphorylated with ATP for 10 s and denatured with TCA. Start of spectra accumulation 0 (a), 30 (b), and 200 min (c) after addition of pepsin. (d–f) Control sample of unphosphorylated sarcoplasmic vesicles. Phosphorylation has been prevented by addition of EGTA. Start of spectra accumulation 0 (d), 30 (e), and 200 min (f) after addition of pepsin. Line broadening of 20 Hz by exponential multiplication of the time domain data, 4000 accumulations per spectrum, temperature during the measurement 288 K.

prepared. The NMR spectrum still exhibits signal X which disappears after successive addition of hydroxylamine (Figure 1d–f).

Phosphoenzyme. All attempts to directly detect the ^{31}P NMR signal of the phosphoenzyme in spectra of native vesicles failed probably because the signal was broadened beyond the detectability against the intense background of phosphorus signals of membrane phospholipids and of impurities containing phosphorus atoms. For increasing the chance for detecting phosphoenzyme intermediates, we prepared fragments of the sarcoplasmic ATPase. The ^{31}P NMR signals should have a smaller line width and should be easily detectable. After digestion with Pronase as described by Bastide et al. (1973), no NMR signal originating from phosphopeptides of the sarcoplasmic ATPase could be detected probably because most of the phosphoenzyme decomposed during the digestion procedure at neutral pH.

Since the phosphointermediate is known to be acid stable, we performed the digestion at low pH using pepsin having a pH optimum between pH 1.8 and 2.2.

Figure 2 shows the spectrum obtained for sarcoplasmic ATPase phosphorylated by ATP. After digestion with pepsin at 288 K for 30 min, a new signal P (labeled by an arrow) showed up at −6.5 ppm. The control sample (Fig. 2 d–e) has been prepared in essentially the same way but the phosphorylation of the ATPase has been inhibited by addition of EGTA. As to be expected signal P cannot be observed under

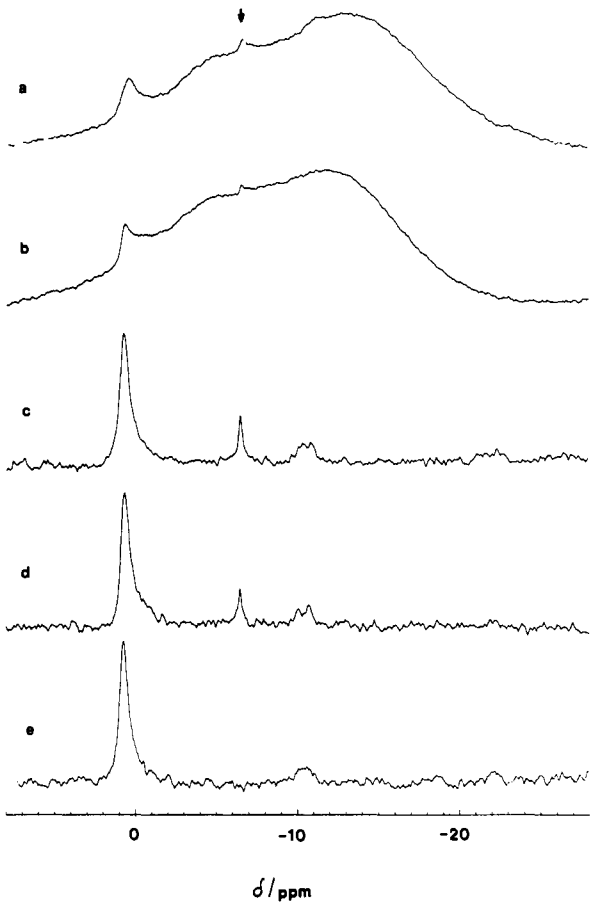

FIGURE 3: Distribution of peptic fragments of sarcoplasmic ATPase phosphorylated with ATP. ^{31}P NMR spectra of (a) a sample which has been prepared as described in Figure 2a (spectra were recorded 200 min after addition of pepsin), (b) the pellet of sample (a) after centrifugation at 30000 rpm for 20 min, rehomogenized in 30 mM HCl, (c) the supernatant of sample (a) after centrifugation at 30000 rpm for 20 min, (d) sample (c) immediately after addition of hydroxylamine (200 mM), and (e) sample (d) after 12 h at room temperature. Line broadening of 20 Hz by exponential multiplication of the time domain data, 4000 accumulations per spectrum, temperature during the measurement 288 K.

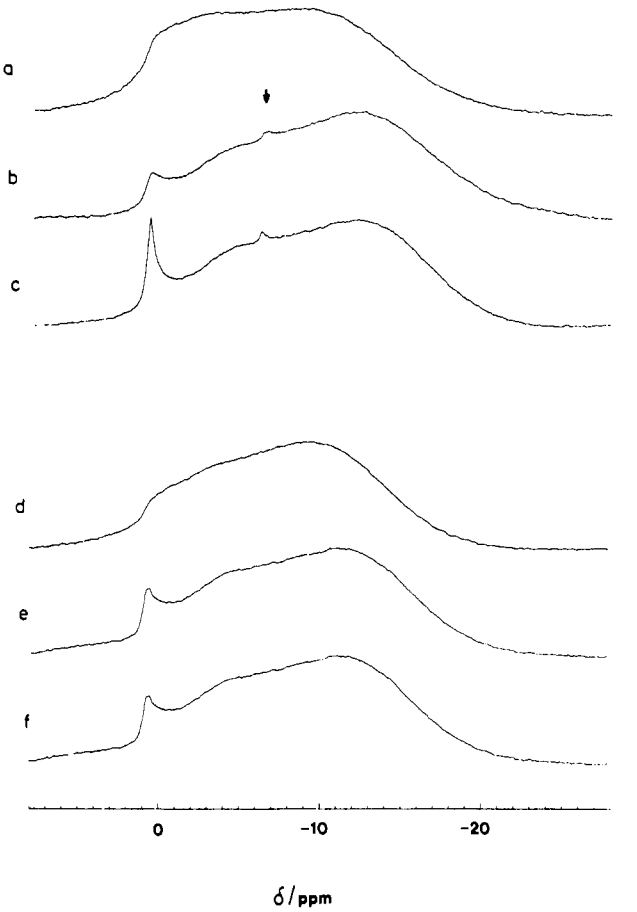

FIGURE 4: ^{31}P NMR spectra of peptic fragments of sarcoplasmic ATPase phosphorylated with inorganic phosphate. (a–c) Sarcoplasmic vesicles phosphorylated with P_i and denatured with TCA. Start of spectra accumulation 0 min (a), 11 (b), and 23 h (c) after addition of pepsin. (d–f) Control sample of unphosphorylated sarcoplasmic vesicles. Phosphorylation has been prevented by omitting inorganic phosphate. Start of spectra accumulation 0 min (d), 90 min (e), and 12 h (f) after addition of pepsin. Line broadening of 20 Hz by exponential multiplication of the time domain data, 4000 accumulations per spectrum, temperature during the measurement 288 K.

these conditions. The same is true when the phosphorylation of the sarcoplasmatic ATPase is prevented not by addition of EGTA but by omitting ATP (spectra not shown). In phosphorylated preparations, signal P can be observed for more than 12 h at pH 2 and 278 K and disappears after approximately 48 h. At pH values higher than pH 4, signal P decays rapidly.

After centrifugation, signal P can be observed in the supernatant as well as in the rehomogenized pellet (Figure 3). The intense signal of the phospholipids is not contained in the spectrum of the supernatant.

Besides signal P, at least two additional resonances can be observed, signal A at 0.5 ppm and signal Q at −10.5 ppm. As the variation of pH shows, signal A is not homogeneous but consists of at least two components with chemical shift and pK values typical for inorganic phosphate and the phosphoseryl residues of pepsin (Edmondson & James, 1979). The incubation with hydroxylamine leads to a faster disappearance of signal P but has virtually no influence on signal A or signal Q (Figure 3).

Essentially the same experiments as described above were performed by phosphorylating the sarcoplasmic ATPase with inorganic phosphate. In this case, a signal P′ can be observed which shows the same properties as signal P obtained after phosphorylation with ATP (some of the experimental results are shown in Figure 4). Again, signal P′ cannot be observed under conditions where no phosphorylation is to be expected, e.g., in the absence of inorganic phosphate (Figure 4d–f). Mixing two samples, one containing fragments of sarcoplasmic ATPase phosphorylated with ATP and one containing fragments of sarcoplasmic ATPase phosphorylated with P_i, shows that signals P and P′ cannot be distinguished by phosphorus NMR under various conditions, implying that the correspondent phosphorylated compounds are most probably identical.

No attempt has been made to determine the absolute concentrations of the phosphointermediates from the NMR spectra, because this is generally rather problematic if the spectroscopic conditions are far from ideal (no internal standard, superpositions and base-line distorsions, limited signal-to-noise ratio). However, a lower limit for detection is given by the signal-to-noise ratio obtainable; i.e., the concentrations of phosphointermediates detected are probably on the order of 0.1 mM.

The dependence of the chemical shifts of resonances P and P′ has been followed as function of pH. The curve depicted in Figure 5 represents the theoretical curve obtained from a nonlinear least-square fit of the experimental data. The parameters obtained from this procedure, chemical shift δ_1 at low-pH values, chemical shift δ_2 at neutral pH, and pK values, are summarized in Table I together with the data from some

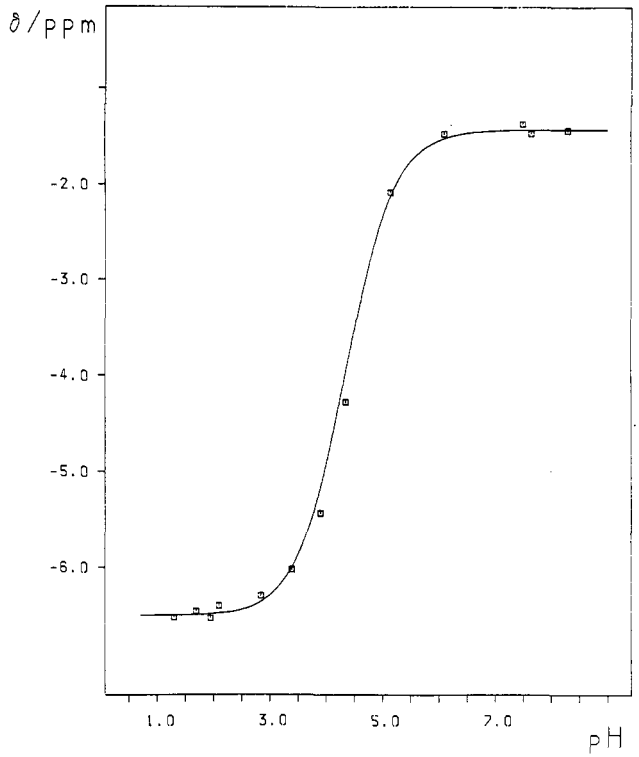

FIGURE 5: pH dependence of chemical shift of the ^{31}P NMR signal of peptic fragments of phosphorylated sarcoplasmic ATPase. The curve depicted has been calculated with the parameters given in Table I obtained from a nonlinear least-squares fit of the data.

Table I: pH Dependence of Chemical Shifts δ_1 and δ_2 in Peptic Fragments of Phosphorylated Sarcoplasmic (Ca^{2+},Mg^{2+})-ATPase

compound	δ_1	δ_2	pK
phosphorylated sarcoplasmic ATPase	−6.52 ± 0.02	−1.42 ± 0.02	4.3 ± 0.2
Gly-Gly-(β-phospho-Asp)-Ala[a]	−6.8	−1.4	5.0
β-phospho-Asp[a]		−1.6 (pH 6.8)	
acetyl phosphate[b]		−2.3 (pH 6.7)	
β-phospho-Asp[c]		−13.2 (pH 7.4)	
Ser-(β-phospho-Asp)[c]		−19.0 (pH 7.4)	

[a] Experimental and spectroscopic details will be published elsewhere (G. M. Sontheimer and H. R. Kalbitzer, unpublished results). [b] Values taken from Bock et al. (1975) in our sign convention (see Materials and Methods). [c] Values taken from Fossel et al. (1981) adapted to our reference system by adding 1.6 ppm and changing the sign (see Discussion).

model compounds containing β-phosphoaspartyl groups.

Discussion

Reinvestigation. Fossel et al. (1981) describe a new ^{31}P NMR signal at +17.5 ppm after phosphorylation of sarcoplasmic vesicles with ATP which they assigned to the β-phosphoaspartyl residue of phosphorylated (Ca^{2+},Mg^{2+})-ATPase. With our sign convention and comparing the resonance positions of the resonances of free ATP, this corresponds to −19.1 ppm in our reference scale, which is exactly the frequency of resonance X. As shown in Figure 1, resonance X can be observed in the supernatant after centrifugation of the solution, which contains no or almost no sarcoplasmic ATPase, as well as in solutions of ATP and metal ions which have never had contact with sarcoplasmic ATPase. Undoubtedly, signal X in the protein-free preparation originates from the β-phosphate of free Mg·ATP, which is in slow exchange in the NMR time scale with free ATP at low temperatures and high magnetic field (Sontheimer et al., 1986). The same is true in the presence of vesicles using experimental conditions identical with those described by Fossel et al. (1981). Another observation which they claimed supported their assignment was the disappearance of the corresponding resonance line after addition of hydroxylamine, an agent which is supposed to cleave the phosphoaspartyl bond. However, as can be seen in Figure 1, the same phenomenon can be observed for the signal of free Mg·ATP which disappears after addition of hydroxylamine. Most probably, this is due to an interaction of hydroxylamine with the phosphate groups of ATP, causing a decrease of the apparent binding constant for magnesium (and possibly an increase of the off-rate), analogous to the interaction of other monoamines with ATP (Sapper et al., 1980).

The position of the assumed phosphoaspartyl resonance is extremely unusual for acyl phosphates, a fact Fossel et al. (1981) also noted. Therefore, they studied two model compounds they synthesized, β-phosphoaspartic acid and seryl-β-phosphoaspartic acid. The obtained chemical shift values of +11.6 and +17.4 ppm (in our reference system, approximately −13.2 and −19 ppm) appeared to agree better with +17.5 ppm found for the ATPase than −1.5 to −2.3 ppm reported for the model compound acetyl phosphate (James et al., 1985). In contrast to these findings, recent studies on β-phosphoaspartic acid and the model peptide Gly-Gly-(β-phospho-Asp)-Ala gave chemical shift values near −1.4 ppm at neutral pH (Table I).

All evidence taken together, the signal assigned to the phosphointermediate of sarcoplasmic ATPase by Fossel et al. (1981) represents most probably the signal of free Mg·ATP. In the same paper, they describe a signal they assigned to the phosphointermediate of the (Na^+,K^+)-ATPase. The same arguments as above appear to apply to this enzyme; i.e., the described signal probably represents also the signal of free Mg·ATP. The only observation which cannot be explained as a signal from free Mg·ATP is the occurrence of a signal at +17.6 ppm (in our reference system, −19.2 ppm) in the presence of P_i, magnesium, and ouabain in the absence of ATP. So far, we have no trivial explanation for this observation, but at least the signal described cannot originate from a simple β-phosphoaspartyl residue as the reinvestigation of the model peptide shows. The only molecules which are not unlikely to be present in the sample and which have chemical shift values in the appropriate range are polyphosphates. However, the published shift values between −22 and −24 ppm (Waki & Hatano, 1982; James, 1985) do not agree very well with the reported shift value.

Phosphointermediate of the Sarcoplasmic ATPase. It has been assumed earlier but never shown experimentally that phosphorylation by ATP leads to the same product as phosphorylation by P_i. In fact, it could be possible that two chemically distinct phosphointermediates could result from the phosphorylation by ATP and P_i. The digestion experiments described above show clearly that phosphorylation with either ATP or P_i results in the same phosphorylation product in fragmented ATPase. The pH dependence of chemical shifts permits the identification of the chemical nature of the phosphointermediate; from comparison with model compounds, it can be identified as a β-phosphoaspartyl residue. This confirms the results obtained by chemical methods for the phosphorylation with ATP (Bastide et al., 1973), although both methods do not exclude completely that in the native protein different phosphorylation products may exist.

The pK value in the fragments is almost 1 ppm value lower than that found in the model peptide Gly-Gly-Asp-Ala. One

explanation for the increase in pK would be the interaction with a positively charged group of the fragment, e.g., with the side chain of the lysyl residue which is the next neighbor of phosphoaspartate in the sequence.

ACKNOWLEDGMENTS

We thank A. Pfandke for preparing the sarcoplasmic vesicles. We are grateful to the Bruker AG, Karlsruhe, for permitting us to use the AM-400 spectrometer and to Dr. Spraul, Dr. Bermel, and Dr. Kuhn for their help on this instrument.

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Fourier Transform Infrared Investigation of the *Escherichia coli* Methionine Aporepressor†

P. W. Yang and H. H. Mantsch*
National Research Council of Canada, Ottawa, Ontario, Canada K1A 0R6
J. L. R. Arrondo‡
The University of the Basque Country, 48080 Bilbao, Spain
I. Saint-Girons, Y. Guillou, G. N. Cohen, and O. Bârzu
Institut Pasteur, 75724 Paris, France
Received September 15, 1986; Revised Manuscript Received December 4, 1986

ABSTRACT: This study represents the first physicochemical analysis of the recently cloned methionine repressor protein (Met aporepressor) from *Escherichia coli*. Infrared spectrometry was used to investigate the secondary structure and the hydrogen–deuterium exchange behavior of the *E. coli* Met aporepressor. The secondary structure of the native bacterial protein was derived by analysis of the amide I mode. The amide I band contour was found to consist of five major component bands (at 1625, 1639, 1653, 1665, and 1676 cm^{-1}) which reflect the presence of various substructures. The relative areas of these component bands are consistent with a high α-helical content of the peptide chain secondary structure in solution (43%) and a small amount of β-sheet structure (7%). The remaining substructure is assigned to turns (10%) and to unordered (or less ordered) structures (40%). The temperature dependence of the infrared spectra of native Met aporepressor in D_2O medium over the temperature interval 20–80 °C indicates that there are two discrete thermal events: the first thermal event, centered at 42 °C, is associated with the hydrogen–deuterium exchange of the hard-to-exchange α-helical peptide bonds accompanied by a partial denaturation of the protein, while the second event, centered around 50 °C, represents the irreversible thermal denaturation of the protein.

DNA binding proteins, acting specifically as activators or inhibitors of transcription of operons, have been investigated extensively by use of genetic and biochemical approaches (Takeda et al., 1983). The methionine (Met) aporepressor, the polypeptide product of the *MetJ* gene, is a regulatory protein that is required for methionine repression of the methionine regulon (Holloway et al., 1970; Kung et al., 1972; Saint-Girons et al., 1984). Genetic evidence and recent in vitro experiments with the purified Met aporepressor have shown that *S*-adenosylmethionine is the corepressor of the methionine regulon (Greene et al., 1970, 1973; Schoeman et al., 1985; Saint-Girons et al., 1986).

Several properties of the Met aporepressor protein make it attractive for physicochemical studies. It is a small, dimeric protein, each polypeptide chin consisting of 104 amino acid residues, whose primary structure has been deduced from the gene sequence (Saint-Girons et al., 1984). Each monomer binds 1 mol of *S*-adenosylmethionine in an apparently noncooperative manner, suggesting that the two corepressor

† Issued as NRCC No. 25983.
‡ Recipient of a NATO research fellowship.

0006-2960/87/0426-2706$01.50/0