$\simeq$ 2.5 kcal/mol. In addition, if we write G^M and G^{MO_2} as the absolute free energy of a deoxy- or oxymyoglobin, then the free energy of O_2 binding is $\Delta G^M = G^{MO_2} - G^M$ and $\delta(\Delta G) = \Delta G^{Co} - \Delta G^{Fe} = (G^{CoO_2} - G^{FeO_2}) - (G^{Co} - G^{Fe})$, where the first parenthesis represents the free-energy difference between oxy-CoMb and oxy-Mb and the second, the free-energy difference between CoMb and Mb. Similar equations hold for enthalpy and entropy. The constancy of $\delta(\Delta G)$, $\delta(\Delta S)$, and $\delta(\Delta H)$ suggests that the thermodynamic differences between Mb and CoMb and between MbO_2 and $CoMbO_2$ are also constant.

A corollary to this conclusion is that it should be equally proper to draw inferences concerning protein influences upon O_2 binding by use of comparisons with either cobalt or iron porphyrins as model compounds. This is of some importance because it is relatively easy to measure solution O_2 binding by a five-coordinate cobalt porphyrin, but difficult to do so for iron porphyrins; in the presence of an excess of nitrogenous base, a typical ferrous porphyrin is six-coordinate, and one can only readily measure the thermodynamics of the replacement of a base by O_2.[23] Cobalt porphyrins are readily prepared in the five-coordinate state, with a single axial base, and thus it is possible to directly observe the addition of O_2, just as occurs in myoglobins.[24]

The "picket fence" porphyrins of Collman et al. are the only model compounds for which data for oxygen binding to a five-coordinate metal are available for both Fe and Co.[8] Comparisons of the $\delta(\Delta S)$ and $\delta(\Delta H)$ of the model system with those in the proteins reveal a substantial difference in the oxygen binding environments of proteins and model. In particular, the difference between $\delta(\Delta S) \simeq 12$ eu for the am proteins and $\delta(\Delta S) \simeq 0$ eu for the model system is intriguing. Collman et al.[5,8] stated that the essentially unchanged ΔS of binding of *all* their models ($\Delta S = 53 \pm 3$ eu, standard state of 1 Torr) can be completely accounted for, on statistical mechanical grounds, by the entropy change calculated for the loss of translational and rotational entropy of bound O_2. The positive $\delta(\Delta S)$ found in the protein then argues that there must be other processes involved, and that metal exchange makes a greater difference in the proteins than in the models. This could be one quantitative measure that nature has achieved selectively for the central metal ion in these proteins.

The exact values of $\delta(\Delta S)$ and $\delta(\Delta H)$ reported here are not particularly important, for they are subject to possible systematic differences in the measurements of the FeMb and CoMb. Nevertheless, these results do indicate that if one looks in proper detail, the oxygen-binding properties of a prosthetic group incorporated within an apoprotein can differ significantly from those of the model. Such a conclusion would also arise from looking to nonmammalian O_2 carriers;[25] indeed, we would expect a larger group of proteins to exhibit a wide range of behaviors. The protein is more than a mere heme container.

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Communications to the Editor

A 360-MHz Photo-CIDNP Study of Bovine Pancreatic Phospholipase A_2. Observation of a pH-Dependent Conformational Change

Sir:

The photo-CIDNP[2] technique, recently applied to biological macromolecules,[3-6] has proven to be quite useful in the assignment of tryptophan, tyrosine, and histidine residues in the ^1H NMR spectrum of proteins. Selective nuclear spin polarization induced in a small number of surface residues results in a great simplification of the photo-CIDNP difference spectrum compared with the normal NMR spectrum. This has allowed us to detect a pH-dependent conformational change in the N terminus of bovine phospholipase A_2. The CIDNP results are compared with ^{13}C NMR titration data of the same enzyme in which the N-terminal alanine residue has been replaced by 90% enriched L-[3-^{13}C]alanine.

Pancreatic phospholipase A_2 hydrolyzes the fatty ester bond at the 2 position of naturally occurring 3-*sn*-phosphoglycerides in the presence of Ca^{2+} ions.[7] The enzyme is secreted as a single chain precursor which can be converted into the active enzyme by tryptic cleavage of the Arg^{-1}–Ala^1 bond which makes Ala^1 the new N-terminal amino acid.[8,9] In the porcine enzyme the α-NH_3^+ group is thought to interact with a carboxylate situated at the end of a hydrophobic cleft. This interaction in the active enzyme induces a special site, the so-called interface recognition site (IRS) which is responsible for micellar binding. The ionization state of the α-ammonium group regulates this binding.[10,11]

According to Slotboom et al.[12] we have substituted the

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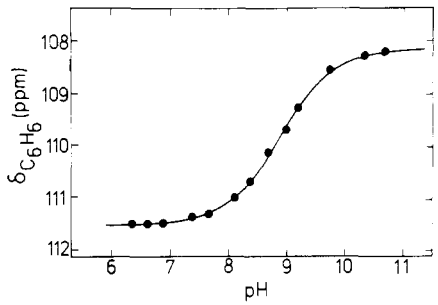

Figure 1. Plot of the ^{13}C chemical shift of the 90% enriched ^{13}C nucleus in bovine [L-[3-^{13}C]-Ala^1)-AMPA as function of pH. The curve has been calculated using a least-squares fitting procedure yielding a pK of 8.9 and a Hill coefficient of 0.98.

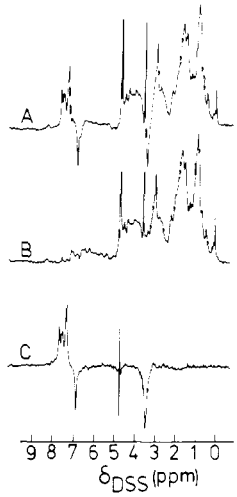

Figure 2. 360-MHz ^1H FT spectra (25 scans) of a D_2O solution of 1 mM bovine phospholipase A_2 and 0.2 mM flavin (in 5 mM Tris at pH 9.3 at 30 °C): A, "light" spectrum obtained by irradiating the sample for 0.6 s by an Argon laser prior to data acquisition; B, "dark" (normal) spectrum; C, photo-CIDNP difference spectrum (A-B). Light and dark free induction decays were taken alternatingly. DSS = sodium 2,2-dimethyl-2-silapentane-5-sulfonate.

N-terminal amino acid in bovine ϵ-amidinated phospholipase A_2 for 90% enriched N-[3-^{13}C]alanine in order to determine the pK of the α-ammonium group. To prevent unwanted incorporation of labels on the ϵ-amino groups of lysine we used the enzymatically active ϵ-amidinated phospholipase A_2 (AMPA). The observed high pK of 8.9 (Figure 1) at 25 °C supports the hypothesis of an ionic interaction between the α-NH_3^+ group and a carboxylate in a hydrophobic environment.[13-15]

In the porcine enzyme the conformational change upon deprotonation of the α-NH_3^+ group (pK = 8.4) can also be monitored by the pH dependency of the fluorescence intensity of the unique Trp residue at position 3 from the N terminus.[16] However, the pK value of 8.9 as reported here for the α-NH_3^+ group of the bovine enzyme cannot be determined accurately by this technique owing to the quenching effect on the Trp fluorescence by the ionization of tyrosine residues above pH 9.

Supplemental evidence for the proposed conformational change concomitant with the deprotonation of the α-amino group can be obtained by the photo-CIDNP method. This method is based on a reversible reaction of a photoexcited dye with exposed aromatic amino acid side chains.[3,4] ^1H NMR lines of these accessible residues are selectively enhanced in the spectrum of the native protein. Figure 2 shows a typical photo-CIDNP experiment of phospholipase A_2 using 3-carboxymethyllumiflavin as a dye. "Light" and "dark" free induction decays were collected by alternatingly irradiating the

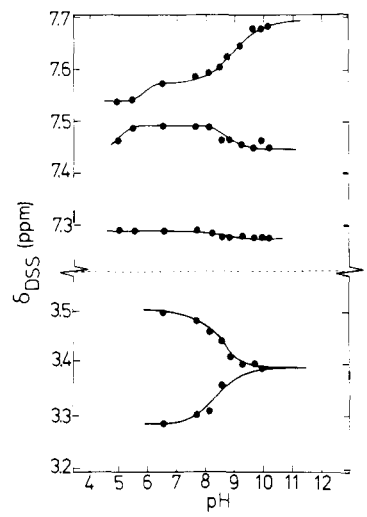

Figure 3. Plot of the ^1H chemical shift (at 360 MHz) of the photo-CIDNP signals of Trp^3 in bovine phospholipase A_2 as function of pH at 25 °C. The upper part of the figure represents resonances from the aromatic C-2, C-4, and C-6 protons of Trp^3, while the curves in the lower part originate from the β-CH_2 protons.

sample in the probe with an argon ion laser. After Fourier transformation this results in the "light" and "dark" spectra (Figures 2A and 2B, respectively). The difference spectrum (Figure 2C) shows the pure CIDNP effect. From model studies[6] it appeared that the positive resonances in the aromatic region originate from the single Trp^3 and the emission line from a tyrosine residue. Using phospholipases A_2 in which tyrosine residues are selectively modified,[17] it can be concluded that the emission line can be assigned to the ortho protons of Tyr.[6,9] The negative resonance at 3.41 ppm belongs to the β-CH_2 protons of tryptophan.[5]

The CIDNP spectrum of Trp^3 shows important changes in the pH region from 8 to 10. One aromatic proton of Trp^3 (probably C-2) is shifted 0.11 ppm to lower field while another proton (probably C-4) shifts 0.04 ppm to higher field. Furthermore, the two β protons of Trp^3 which were not equivalent at neutral pH shift to the same position (3.39 ppm). These shifts have been depicted as function of pH in Figure 3. The aromatic protons of Trp^3 titrate with a pK of 8.9 reflecting thus the deprotonation of the α-NH_3^+ group of Ala^1.

Therefore from ^{13}C NMR and ^1H CIDNP results it may be clear that the deprotonation of the N-terminal Ala^1 is accompanied by a conformational change which can be monitored by Trp.[3] Upon deprotonation of the N-terminal amino acid the salt bridge is disrupted and as a result the N terminus is forced to leave the interior of the protein.

The present study shows a useful feature of the photo-CIDNP technique in that a single amino acid residue can be selected and its ^1H shifts monitored, whereas in the normal spectrum the resonances are buried in the aromatic envelop. The method has also been very helpful in the study of protein-substrate interactions, which will be reported shortly.

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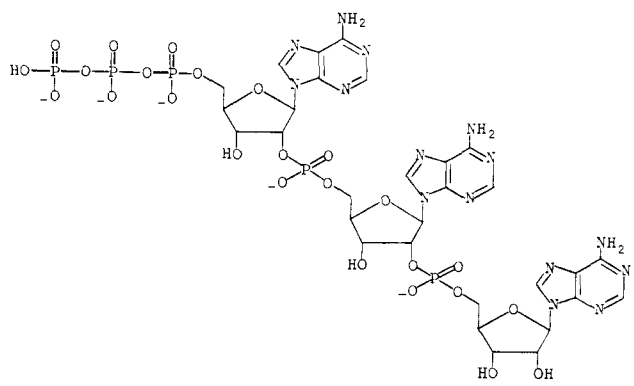
Chemical Synthesis of 5′-*O*-Triphosphoryladenylyl-(2′→5′)-adenylyl-(2′→5′)-adenosine (2-5A)

Sir:

It has recently been reported[1] that a low-molecular-weight inhibitor of cell-free protein synthesis, effective at subnanomolar concentrations, is formed on incubation of extracts from interferon-treated cells or rabbit reticulocytes with double-stranded ribonucleic acids and adenosine 5′-triphosphate. On the basis of its spectroscopic, electrophoretic, and chromatographic properties and of enzyme and hydroxide ion promoted hydrolysis studies, the structure 5′-*O*-triphosphoryladenylyl-(2′→5′)-adenylyl-(2′→5′)-adenosine (2-5A, **1**) has been assigned[1] to the inhibitor. In order to confirm this assignment and provide a source of a compound which could prove to be of much importance in the control of cell metabolism and thus a potentially valuable chemotherapeutic agent, we now report an unambiguous chemical synthesis of 2-5A (**1**). It should be added that the presence both of the terminal triphosphate residue and the unnatural 2′→5′ internucleotide linkages makes 2-5A (**1**) an oligonucleotide derivative of exceptional chemical interest and its successful preparation illustrates the versatility of presently available synthetic methods.



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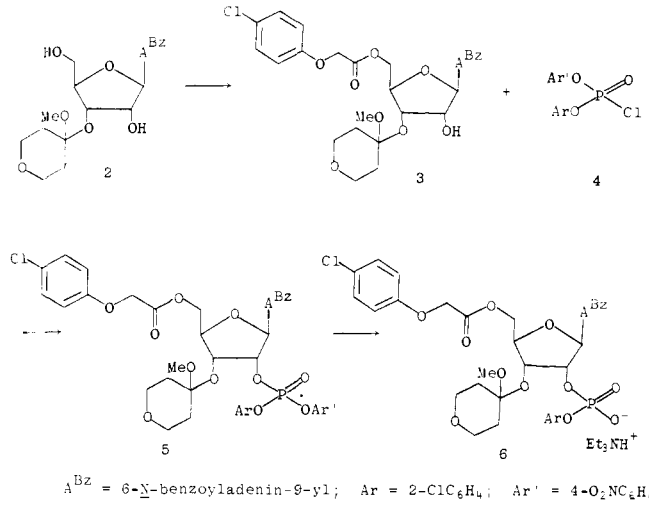
Scheme I



A^{Bz} = 6-*N*-benzoyladenin-9-yl; Ar = 2-ClC_6H_4; Ar′ = 4-$O_2NC_6H_4$

3′-*O*-Methoxytetrahydropyranyl-6-*N*-benzoyladenosine (**2**) was prepared, in four steps, from crystalline 5′-*O*-acetyl-2′-*O*-*tert*-butyldimethylsilyladenosine[2] and isolated as a colorless glass in ~75% overall yield.[3] When **2** was treated with an excess of *p*-chlorophenoxyacetyl chloride in acetonitrile-pyridine solution, 5′-*O*-*p*-chlorophenoxyacetyl-3′-*O*-methoxytetrahydropyranyl-6-*N*-benzoyladenosine (**3**) was obtained and isolated as a colorless crystalline solid in 40% yield.[5] Treatment of **3** with 2-chlorophenyl 4-nitrophenyl phosphorochloridate[6] (**4**, Ar = 2-ClC_6H_4; Ar′ = 2-$O_2NC_6H_4$) in pyridine solution gave the phosphotriester (**5**) (R_F 0.75 (system A)[7]) in 95% isolated yield. When **5** was treated with a tenfold excess of *p*-thiocresol and triethylamine[6] in acetonitrile solution, the triethylammonium salt (**6**) was obtained[8] and isolated as a colorless powder in 91% yield (Scheme I).

A solution of the latter triethylammonium salt (**6**) and a slight excess (~1.1 mol equiv) of 2′,3′-*O*-methoxymethylene-6-*N*-benzoyladenosine (**7**) in anhydrous pyridine solution was treated with an excess (6.5 mol equiv) of 1-mesitylenesulfonyl-3-nitro-1,2,4-triazole[6b,9] (MSNT, **8**). The reaction (Scheme II) was worked up after 2 h and the products were then chromatographed to give the fully protected dinucleoside phosphate in 70% isolated yield. The *p*-chlorophenoxyacetyl protecting group[10] was removed from this material by treating it with 0.2 M sodium hydroxide in dioxane–water (19:21 v/v) for 30 s at 0 °C and the partially protected dinucleoside phosphate (**9**) (R_F 0.55 (system A)[7], 0.33 (system B)[7]) thereby obtained was isolated in 92% yield. The required partially protected trinucleoside diphosphate (**10**) (R_F 0.29 (system B)[7]) was prepared in the same way by allowing **9** (1.0 mol equiv), **6** (1.2 mol equiv), and MSNT (**8**, 7.5 mol equiv) to react together and then removing the *p*-chlorophenoxyacetyl protecting group by alkaline hydrolysis; this material (**10**) was isolated in 75% overall yield, based on **9**.

The fully protected trinucleoside diphosphate, obtained by treating **10** with a twofold excess of 9-phenyl-9-xanthenyl (pixyl) chloride[11] in pyridine solution, was treated with (i) 0.3 M N^1,N^2,N^3,N^3-tetramethylguanidinium *syn*-4-nitrobenzaldoximate[6b] in dioxane–water (1:1 v/v) at 20 °C for 22 h, (ii) aqueous ammonia (*d* 0.88) at 20 °C for 24 h, (iii) 0.01 M hydrochloric acid at 20 °C for 6 h, and (iv) dilute aqueous

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