Binding of Adenosine 5′-Diphosphate to Creatine Kinase. An Investigation Using Intermolecular Nuclear Overhauser Effect Measurements†

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ABSTRACT: Measurements of the nuclear Overhauser effect (NOE), which is a nuclear magnetic resonance (NMR) double resonance technique, for the H-2 proton on ADP have been used to identify the amino acid residue binding ADP at the active site of creatine kinase. Application of a strong radiofrequency field H_2 at a frequency of 0.9 ppm or 1.7 ppm downfield from the proton resonance of 2,2-dimethyl-2-silapentane-5-sulfonate results in a negative NOE for the H-2 proton resonance of ADP in its complex with creatine kinase. The magnitude of the NOE for the ADP H-2 proton depends on the ratio of ADP to creatine kinase binding site concentration; the dependence indicates that there is rapid exchange between free and bound ADP. Comparable values of the NOE for the H-2 proton of adenosine 5′-mono-, 5′-di-, and 5′-triphosphate and inosine 5′-diphosphate in binary complexes with creatine kinase show that the binding site for these nucleotides is the same. The large negative NOE for the H-2 proton of ADP is maintained for the various binary, ternary, quaternary, and pentenary complexes of creatine kinase with ADP formed by addition of the activator Mg(II), the other substrate creatine, and the planar anion nitrate which is an inhibitor. These results indicate that the conformational changes known to occur upon addition of the other ligands do not involve the entire active site. In particular, the environment around the nucleotide is unperturbed. Inactivation of creatine kinase by reaction with iodoacetamide causes considerable conformational changes. However, as indicated by the large negative NOE for the H-2 proton of ADP in a binary complex with the inactivated enzyme, the environment around the base is altered minimally. Experiments were performed to identify the proton groups on the enzyme, resonating at 0.9 and 1.7 ppm, which interact with the ADP H-2 proton. An NOE was not observed when the aromatic protons of the enzyme were irradiated with the strong radiofrequency field H_2 implying that aromatic protons are not near the H-2 proton of ADP in the enzyme complex. The H-2 proton of 1-N^6-ethenoadenosine 5′-diphosphate, an analogue of ADP with the 1-nitrogen and 6-nitrogen blocked from potentially hydrogen bonding, still exhibits a large NOE in the nucleotide–enzyme complex. The indication is that the protons promoting the H-2 proton NOE are not on an amino acid residue which binds ADP by hydrogen binding. Creatine kinase was inactivated by reacting the single essential arginyl residue per subunit with diacetyl. No NOE for the H-2 proton of ADP in the presence of the inactivated enzyme was observed. Observation of the H-2 proton resonance of the inhibitor adenosine in the presence of the enzyme revealed no NOE in contrast to the observations with the adenine nucleotides. On the basis of the chemical shift of the enzyme protons promoting the NOE, dependence of the NOE on nucleotide or nucleoside variations, and lack of an NOE for ADP in the presence of diacetyl-inactivated creatine kinase, it is suggested that the H-2 proton NOE for ADP in its complexes with creatine kinase is due to the β- and γ-methylene protons of an arginyl residue. The positively charged guanidino group of the arginyl residue serves to bind the α-phosphoryl (and possibly the β-phosphoryl) group of the nucleotide.

Nuclear magnetic resonance (NMR[1]) studies have been utilized in an attempt to elucidate the role of specific amino acid residues at the active site of enzymes in solution (Roberts and Jardetzky, 1970; James, 1975). The most fruitful experiments have been carried out with relatively small ($\lesssim$30 000 molecular weight) enzymes; the restriction to small enzymes is due principally to the overlapping lines (i.e., overabundance of data) in the NMR spectrum of a protein. Even a small protein has about a thousand different protons, each of which has a resonance line at a frequency characteristic of the electronic environment of that proton. In addition to more overlapping resonances, larger proteins generally lead to broader resonances, mainly due to the slower motions (i.e., increased correlation times) of the larger macromolecules.

Two general approaches to the problem of overlapping resonances in protein spectra have been (1) to examine only those resonances which are shifted away from the main envelope of resonances, e.g., the C2 proton resonances of histidine residues (Meadows et al., 1968), or (2) to employ isotopic labeling of the protein (Crespi et al., 1972; Huestis and Raftery, 1972). In certain cases it may even be possible to apply the latter technique to larger proteins.

A complementary method for studying substrate-active site chemistry has recently been exploited by James and Cohn (1974). Nuclear Overhauser effect (NOE) measurements were used to reveal the role of a lysyl residue on creatine kinase in the enzyme-catalyzed reaction (James and Cohn, 1974). An NOE may be manifested in the resonance of a nucleus on the substrate when the resonance of a nucleus on the enzyme in very close proximity to the bound substrate nucleus is irradiated with a strong radiofrequency field H_2. Since the phe-

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[1] Abbreviations used are: NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; rf, radiofrequency; IDP, inosine 5′-diphosphate; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; DSS, 2,2-dimethyl-2-silapentane-5-sulfonate sodium salt; CK, creatine kinase; EPR, electron paramagnetic resonance; ADP H-2 NOE, nuclear Overhauser effect for the H-2 proton of ADP; etheno-ADP, 1-N^6-ethenoadenosine 5′-diphosphate.

nomenon has an r^{-6} dependence on the distance r between the two nuclei, an NOE will be observed with the substrate nucleus only if the frequency of the H_2 field corresponds to the resonance frequency of a nucleus at the active site of the enzyme. In favorable cases, only one or two groups of equivalent nuclei on the enzyme will promote an NOE for a nucleus on a bound substrate; the resonance frequencies for these few groups of nuclei will be characteristic. This is to be contrasted with the plethora of resonance frequencies exhibited by all the nuclei in the usual NMR spectrum of the enzyme.

In this report the binding of ADP to creatine kinase, which catalyzes the reversible transfer of a phosphoryl group between ATP and phosphocreatine, is examined.

$$\text{ATP} + \text{creatine} \overset{\text{Mg(II)}}{\rightleftharpoons} \text{ADP} + \text{phosphocreatine} + \text{H}^+ \quad (1)$$

It might be noted that the amino acyl residues on the enzyme which bind ADP are particularly important since the evidence from water proton relaxation rate measurements indicates that ADP acts as a bridge between the enzyme and the requisite metal ion activator (O'Sullivan and Cohn, 1968).

Theory

The nuclear Overhauser effect measurement is a double resonance experiment (Noggle and Schirmer, 1971; James, 1975). One nuclear resonance is irradiated with a strong rf field H_2 while another resonance is monitored with the usual weak observing rf field H_1. A change in the intensity of the monitored resonance upon irradiation of the second nuclear resonance with the H_2 field is a manifestation of the nuclear Overhauser effect. The intensity change results from alterations in the populations of the nuclear energy levels brought about by dipolar coupling of the two nuclei. Perturbation of the energy level population of one nucleus by strong irradiation of a second nucleus is referred to as dynamic polarization.

For two dipolar-coupled protons, Balaram et al. (1972) used the dipolar transition probabilities derived by Solomon (1955) to obtain an expression for the fractional enhancement $f_{I_2}(I_1)$ of the integrated proton resonance intensity of nucleus I_1 when nucleus I_2 is strongly irradiated:

$$f_{I_2}(I_1) = \frac{5 + \omega^2\tau_c^2 - 4\omega^4\tau_c^4}{10 + 23\omega^2\tau_c^2 + 4\omega^4\tau_c^4} \quad (2)$$

where ω is the angular resonance frequency, and τ_c is the correlation time for the motion modulating the dipolar interaction. The maximum positive enhancement of NOE of 0.5 (i.e., 50%) is obtained in the short correlation time limit. In the case of long correlation times, as occurs in macromolecules or macromolecular complexes, it is apparent from eq 2 that a maximum negative NOE of -1 (i.e., -100%) may result. In fact, negative NOE's have been observed both for a hormone bound to neurophysin by Balaram et al. (1973) and for formate in a MgADP–creatine–formate complex of creatine kinase by James and Cohn (1974).

Observation of an NOE requires the two irradiated nuclei to be dipolar coupled and in close proximity. The observed NOE is diminished if the monitored nucleus relaxes by any relaxation mechanism other than nuclear dipole–dipole interactions. The dynamic polarization (NOE) due to any particular nucleus which is dipolar coupled to the polarized nucleus being monitored also will be diminished if there is more than one nucleus coupled to that polarized nucleus. In addition it is evident from eq 2 that there will be a range of correlation times for which neither the positive terms nor the negative term in the numerator dominate, and the observed NOE will be inherently small; indeed, it will be zero when $\omega\tau_c = 1.12$.

It is therefore not possible to know a priori that an NOE will be observed for any particular enzyme–substrate complex. Certain conditions, however, are conducive to observation of an intermolecular NOE. (a) The monitored nucleus should not be physically near any other nucleus on the same substrate which possesses a substantial magnetic dipole moment. (b) A deuterated solvent should be employed and deuterium exchange carried out with the exchangeable protons on the enzyme. At the same concentration, deuterium is only 6% as effective as a proton in promoting relaxation. (c) Paramagnetic species need to be excluded from the solution. In particular, that means paramagnetic ions, but it may also prove beneficial to remove paramagnetic O_2. (d) The monitored nucleus ideally should not be strongly scalar coupled to a nucleus with a large quadrupole moment such as ^{14}N since there is a possibility the NOE may be diminished by scalar relaxation of the second kind. (e) If an excess of substrate is present in solution, the rate of exchange between free and bound substrate must be greater than the spin–lattice and spin–spin relaxation rates of the nucleus in the free or bound state. If exchange is rapid, the observed nuclear resonance for the substrate will be a weighted average for three populations: (i) completely relaxed (depolarized) nuclei on substrate molecules in the free state; (ii) nuclei in the bound state being polarized by the nearby irradiated nucleus on the enzyme; and (iii) nuclei in the free state which have not yet relaxed from their polarization while in the bound state. (f) As a consequence of the observed peak being a weighted average of the three populations, a sufficiently low substrate-to-enzyme ratio must be used.

Experimental Procedure

Materials. The back and leg muscles of freshly killed rabbits were used as the source of creatine kinase which was isolated and assayed as described by O'Sullivan and Cohn (1966). Reagents used were sodium ADP (P-L Biochemicals), sodium ATP (Sigma), the sodium salt of 1-N^6-ethenoadenosine 5′-diphosphate (P-L Biochemicals), sodium AMP (P-L Biochemicals), sodium IDP (P-L Biochemicals), adenosine (Nutritional Biochemicals), creatine (Sigma), magnesium acetate (J. T. Baker Chemical), sodium nitrate (Merck), iodoacetamide (Sigma), diacetyl (Fisher), sodium borate (J. T. Baker Chemical), Bio-Gel P-4 (Bio-Rad), Hepes (Sigma), and deuterium oxide (Aldrich).

Enzyme solutions for the NOE studies were prepared by dialyzing the solution against a K^+–Hepes buffer (pD 8.2) in D_2O at least six times. The final concentration of K^+–Hepes was 1 mM. The pD value denotes the meter reading on a Corning Model 10 pH meter standardized against buffers at pH 4 and 7.

Enzyme Modifications. The reaction of the sulfhydryl groups of creatine kinase with iodoacetamide was carried out according to the description of Quiocho and Thompson (1973). The residual specific activity of the enzyme was 2.6 as compared with a specific activity of about 60 for the native enzyme.

The essential arginyl residues (one per subunit) of creatine kinase were reacted with diacetyl in a 50 mM sodium borate buffer, pH 8.3, as described by Borders and Riordan (1975), except that the diacetyl trimer [see Yankeelov et al. (1968)] was employed. The diacetyl-inactivated enzyme preparation had a residual activity of 1.3.

NOE Measurements. All proton NOE measurements were performed at 360 MHz on the Bruker HXS-360 located in the Stanford Magnetic Resonance Laboratory. Several (8–128

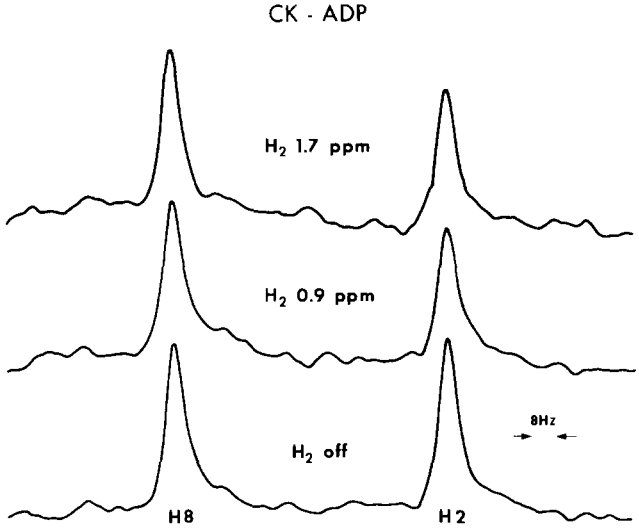

FIGURE 1: The 360-MHz proton NMR resonance lines for the H-8 and H-2 protons on ADP in a sample composed of 63 mg/ml creatine kinase and 20.0 mM ADP in deuterium oxide buffered at pD 8.2 with 1 mM Hepes. The effect of applying the strong rf field H_2 at frequencies 1.7 or 0.9 ppm downfield of DSS is illustrated. Each spectrum is an average of eight scans.

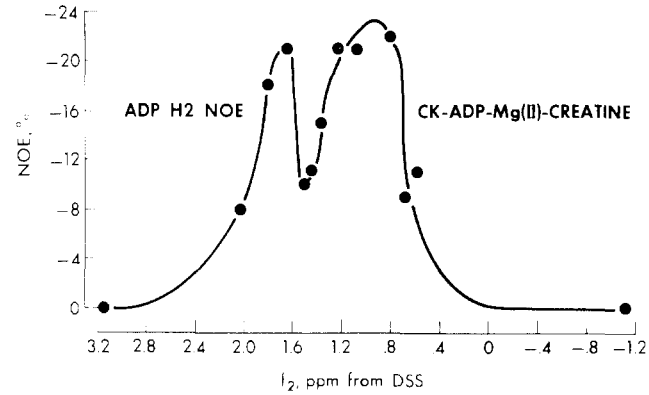

FIGURE 2: The NOE calculated for the H-2 proton of ADP as a function of the frequency of the strong rf field H_2. Although not shown in the plot, a 0% NOE was maintained down to a frequency of 7.6 ppm. The sample consisted of 63 mg/ml creatine kinase, 20.0 mM ADP, 12.2 mM magnesium acetate, and 10.0 mM creatine in a deuterium oxide solution buffered at pD 8.2 with 1 mM Hepes.

scans of the H_1 field, depending on the sample) spectra obtained in the continuous wave mode were averaged on the Nicolet 1080 computer interfaced with the spectrometer while a strong H_2 field was applied at a fixed frequency. As shown in Figure 1, a decrease in the intensity of the H-2 proton peak of ADP in the creatine kinase–ADP complex is obtained when the frequency of the H_2 field is adjusted to 0.9 or 1.7 ppm downfield of the internal reference DSS. For some of the later experiments, rapid scan correlation spectroscopy (Dadok and Sprecher, 1974; Gupta et al., 1974) was employed with considerable time savings since the spectra were scanned at the rate of 235 Hz/s. One solution was checked and found to have the same NOE with correlation spectroscopy as with the conventional continuous wave technique.

Measurement of an NOE entails measurement of peak intensities (i.e., peak areas). However, it was discovered in some of the early experiments on the ADP NOE that the negative NOE calculated from the intensity decrease was equal to the NOE calculated from the peak amplitude decrease, within experimental error. All subsequent NOE measurements involved amplitudes only. It was further observed that the resonance line intensities did not vary, within experimental error, from their intensities in the absence of an H_2 field when the H_2 field was applied at a frequency 1–2 ppm upfield of DSS. In practice NOE values were calculated from the difference between peak amplitudes with the H_2 field at a given frequency and the H_2 field offset to 1–2 ppm upfield of DSS. An NOE value reported here is an average of at least three individual measurements (8–128 scans per measurement) made on the same sample at a temperature of 25 ± 2 °C. Relative errors in the NOE measurements are estimated to be as large as 10% in some cases, depending on the signal-to-noise ratio.

For a few of the experiments, a higher power level was used for the strong H_2 field. In those cases, it was observed that the amplitude of the H-8 proton resonance also decreased (compare with Figure 1). Although the ratio of H-8 to H-2 peak intensities was consistent with the low power experiments, the reported values for the H-2 proton NOE were diminished by the NOE calculated for the H-8 proton in those cases. It was found for a creatine kinase–ADP complex that this correction procedure yielded an NOE of −19 to −21% for both high and low H_2 power levels. A possible explanation for the additional H-2 peak amplitude decrease is that application of H_2 at 0.9 ppm with high H_2 levels will result in a significant H_2 field at 1.7 ppm (or vice versa) which will contribute to a further decrease in peak amplitude.

Results

Nuclear Overhauser Effect for ADP. As illustrated in Figure 1, the intensity of the H-2 proton resonance of ADP (located 8.5 ppm downfield from DSS) in a creatine kinase–ADP complex diminishes when the sample is irradiated with rf field H_2 at a frequency 0.9 or 1.7 ppm downfield from DSS. It should be noted that application of H_2 at 0.9 or 1.7 ppm with a sample containing only ADP in the buffer solution did not produce a measurable NOE. In contrast, the H-8 resonance of ADP in the complex does not decrease in intensity irrespective of the frequency of the H_2 field as that frequency is varied across the entire proton NMR spectrum of creatine kinase. It is quite possible that too many different protons contribute to the dipolar relaxation of the ADP H-8 proton for any *one* proton to promote an appreciable NOE. In fact, NOE experiments with nucleosides (Hart and Davis, 1969a,b) imply that intramolecular contributions from the H-1′, H-2′, H-3′, and H-5′ protons may all be significant for H-8.

Results for the measured NOE of the ADP H-2 proton resonance as a function of the frequency, f_2, of the H_2 rf field are shown in Figure 2 for a creatine kinase–ADP–Mg(II)–creatine complex. Within experimental error, no measurable NOE was detected at any other frequency up to 8.2 ppm. It seems apparent from the results of Figure 2 that two different protons (or groups of equivalent protons) on the enzyme which resonate at 0.9 and 1.7 ppm give rise to the ADP H-2 NOE. The frequencies, 0.9 and 1.7 ppm, do not correspond to the resonance frequency of any protons on the substrates ADP or creatine. The two protons (or groups of protons) therefore are located on the enzyme and are near the ADP H-2 proton in the creatine kinase complex.

The ADP H-2 proton NOE depends on the ratio of ADP to creatine kinase concentration as shown in Figure 3 for the enzyme–ADP–Mg(II)–creatine–nitrate complex. With the samples used in the present studies, the ADP concentration is greater than the creatine kinase binding site concentration and, except at low ADP to enzyme ratios, the NOE is linearly dependent on the ratio for data obtained with H_2 at either 0.9 or

TABLE I: Effect of Mg(II) Activator, Substrates, and Nitrate on the ADP H-2 NOE of Creatine Kinase–ADP Complexes.[a]

			NOE, %	
Mg(II)	Creatine	Nitrate	H_2 at 0.9 ppm	1.7 ppm
−	−	−	−20	−19
+	−	−	−17	−19
−	+	−	−19	−19
+	+	−	−22	−21
+	+	+	−19	−22

[a] Solutions of 63 mg/ml creatine kinase and 20.0 mM ADP in deuterium oxide were buffered at pH 8.2 with 1 mM Hepes. Additions were made to bring the concentration of magnesium acetate to 12 mM, creatine to 10 mM, and sodium nitrate to 0.7 mM.

TABLE II: H-2 NOE for Binary Complexes of Nucleotides and Nucleosides with Creatine Kinase.[a]

	NOE, %	
Nucleotide or Nucleoside	H_2 Applied at 0.9 ppm	1.7 ppm
ADP[b]	−20	−19
IDP[c]	−23	−25
ATP[d]	−22	−24
AMP[e]	−28	−25
Etheno-ADP[f]	−17	−12
Adenosine[g]	−2	0

[a] All solutions were prepared in deuterium oxide with buffering at pD 8.2 with 1 mM Hepes. [b] With 63 mg/ml enzyme plus 20.0 mM ADP. [c] With 61 mg/ml enzyme plus 17.8 mM IDP. [d] With 61 mg/ml enzyme plus 14.3 mM ATP. [e] With 61 mg/ml enzyme plus 17.8 mM AMP. [f] With 60 mg/ml enzyme plus 14.7 mM etheno-ADP. [g] With 77 mg/ml enzyme plus 15.0 mM adenosine.

1.7 ppm. This observation indicates that rapid exchange of free ADP and bound ADP is occurring, and that the measured NOE is a weighted average of the bound and free ADP populations. The observed leveling off of the NOE at low ADP to binding site ratios probably occurs because the residence time of an ADP molecule in the free state is not long enough for appreciable relaxation (depolarization) of the H-2 proton to take place before the molecule is bound to the enzyme and the H-2 proton again is subjected to dynamic polarization via spin exchange with the H_2 irradiated proton on the enzyme.

Dependence of the ADP H-2 NOE on the Presence of Substrate, Activator, and Anion Inhibitor. ADP H-2 NOE measurements were made on samples of the creatine kinase–ADP complex with sequential addition of the requisite divalent metal ion activator Mg(II) (Watts, 1973), the substrate creatine, and nitrate, which is a planar anion inhibitor (Milner-White and Watts, 1971]. The order of addition was changed to produce the various binary, ternary, quaternary, and pentenary complexes. Table I contains the NOE results for these complexes. The results indicate that, with experimental error, the ADP H-2 proton NOE effected by enzyme protons resonating at either 0.9 or 1.7 ppm does not vary as the other substrate, the activator, and the anion inhibitor are added to the complex.

H-2 Proton NOE for Other Nucleotide and Nucleoside Complexes of Creatine Kinase. The NOE's for the H-2 proton of some additional purine nucleotides in binary complexes with

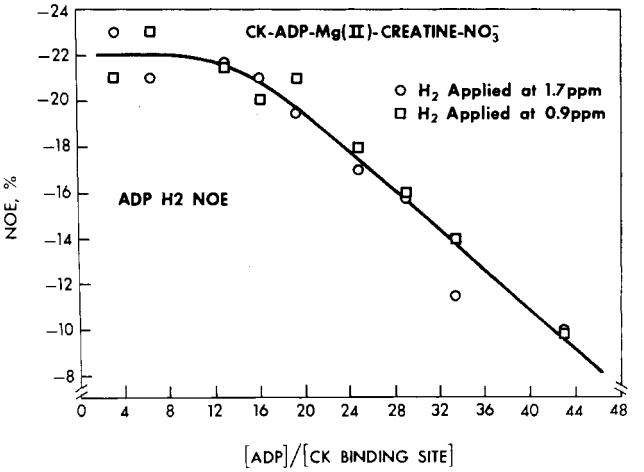

FIGURE 3: The dependence of the NOE measured for the H-2 proton of ADP as a function of the ratio of ADP concentration to creatine kinase binding site concentration. The NOE is calculated with the strong rf field H_2 applied at 1.7 ppm (○) or 0.9 ppm (□) downfield of DSS. The sample is composed of 62 mg/ml creatine kinase, 12.2 mM magnesium acetate, 10.0 mM creatine, 0.7 mM sodium nitrate, and a variable concentration of ADP.

creatine kinase were measured. The results are summarized in Table II.

The NOE for the nucleotide H-2 proton in the enzyme–IDP complex shows, at most, a slightly larger effect than for the enzyme–ADP complexes, and, within experimental error, the NOE values with H_2 applied at 0.9 and 1.7 ppm are the same. Thus, a change in functional group at position 6 of the nucleotide base has a minimal effect. The NOE obtained in the binary enzyme complex with ATP indicates that the binding site for the base moiety of the nucleotide is the same for the reverse reaction as it is for the forward reaction catalyzed by creatine kinase.

It may be noted that the dissociation constants for ATP and IDP from creatine kinase are about an order of magnitude larger than for ADP (James and Morrison, 1966); yet the NOE values are comparable. This illustrates the point that the dissociation constant per se is not the important factor in determining the value of the measured NOE; once a sufficiently large fraction of the ligand is bound, the measured NOE will be solely governed by the rate of relaxation of the H-2 proton on the enzyme-bound ligand.

The ADP analogue 1-N^6-ethenoadenosine 5′-diphosphate (etheno-ADP) forms a binary complex with creatine kinase.[2] The NOE values are lower than with the other nucleotide complexes (see Table II). A major reason for the lower values, however, apparently is due to intramolecular dipolar coupling between the H-2 proton and the nearest etheno proton, since an intramolecular NOE can be measured.

Adenosine is an inhibitor of the reaction catalyzed by creatine kinase (Watts, 1973). Yet, within experimental error, there is no NOE observed for the H-2 proton of adenosine in solutions with adenosine-to-creatine kinase binding site ratios as low as 7.8.

H-2 NOE for ADP with Chemically Modified Creatine Kinase. Reaction of the single sulfhydryl group per subunit of creatine kinase with iodoacetamide inactivates the enzyme. Smith and Kenyon (1974) have demonstrated that the sulfhydryl group is very near the active site but is not essential for

[2] Using the assay procedure of James and Morrison (1966), it was found that etheno-ADP is an inhibitor of the creatine kinase reaction (Schiro and James, unpublished results).

reactivity. A binary complex of the ADP with creatine kinase inactivated by iodoacetamide (54 mg/ml enzyme plus 14.3 mM ADP) still exhibits H-2 proton NOE's of −15% with the H_2 field at 0.9 ppm downfield of DSS and −16% with the H_2 field at 1.7 ppm.

There were reasons to suspect that the proton groups on the enzyme causing the nucleotide H-2 NOE's were the β- and γ-methylene protons of an arginyl residue (see Discussion). Therefore, studies involving modification of arginine in creatine kinase using diacetyl as a reagent were initiated. In the midst of our modification studies, however, Borders and Riordan (1975) published their paper showing (a) that there is one essential arginine per subunit of creatine kinase, (b) that the arginyl residue will react with diacetyl, and (c) the diacetyl reaction is inhibited by the presence of nucleotide. We have found that a sample of ADP with the diacetyl-inactivated enzyme (70 mg/ml enzyme, 15.0 mM ADP) exhibits no NOE ($0 \pm 2\%$) for the nucleotide H-2 proton when the H_2 field is applied at either 0.9 or 1.7 ppm.

Discussion

From the data presented in Figure 2, it can be concluded that two different groups of protons on creatine kinase bring about an NOE for the H-2 proton of ADP in the enzyme complex. The NOE values also do not vary as the divalent metal ion activator Mg(II), the other substrate creatine, and the nitrate inhibitor are sequentially added to the binary enzyme–ADP complex (see Table I). Substrate-induced enzyme conformational changes have been proposed on the basis of the effect of substrates on the reactivity of the cysteyl residue near the active site with sulfhydryl reagents (Watts, 1973). Changes in ligand binding to the divalent metal ion activator necessitating a conformational change at the active site when creatine is added to the enzyme–ADP–Mn(II) complex have been suggested from Mn(II) EPR studies (Reed and Cohn, 1972). Even greater changes in the Mn(II) EPR spectra were observed when planar anions such as nitrate (Reed and Cohn, 1972) or formate (Reed and McLaughlin, 1974) were added to the abortive quaternary complex to form a transition state analogue complex (Milner-White and Watts, 1971). The criticality of this conformational change was demonstrated in the formate NOE investigation of James and Cohn (1974). The enzyme conformation essential for the enzyme-catalyzed reaction to take place exists only when both substrates and the metal ion activator are present. That these conformational changes do not involve the whole active site is demonstrated by the fact that the ADP H-2 NOE effected by either of the two proton groups on the enzyme is indifferent to the presence of Mg(II), creatine, or nitrate in the complex. Although temperature-jump experiments (Hammes and Hurst, 1969) and fluorescent dye studies (McLaughlin, 1974) indicate that a conformational change occurs when ADP is complexed by creatine kinase, once ADP is bound, the active-site conformation in the region of the base apparently undergoes no further alterations. Indeed, the conformational changes that do take place must be fairly subtle since no variation in viscosity or sedimentation constant has been detected (Lui and Cunningham, 1966).

Inactivation of creatine kinase by reaction with the sulfhydryl reagent iodoacetamide is also known to cause significant conformational changes (Watts, 1973). Yet, the −15% NOE observed for the H-2 proton of ADP in a binary complex with the inactivated enzyme indicates that the environment of the base binding site has been altered minimally.

We can hypothesize that the protons leading to the observed NOE's are on amino acid residues involved in binding ADP since the r^{-6} distance dependence of the NOE requires the protons on the enzyme to be very close to the H-2 proton of ADP in the complex. One might postulate a priori that the adenine moiety of ADP is in a pocket of aromatic amino acid residues as suggested for the nucleotide binding site of the dehydrogenases (Rossmann et al., 1974). This possibility cannot be entirely ruled out for ADP binding to creatine kinase. However, no nuclear Overhauser effect was found for the ADP protons in the enzyme complex when the strong H_2 field was applied at frequencies corresponding to aromatic proton resonances.

TABLE III: Protons on Amino Acid Residues Possibly Leading to the Observed ADP H-2 Proton NOE.[a]

Proton Resonance	Chemical Shift
Lys γ-CH_2	1.4
Lys β- and δ-CH_2	1.7
Arg γ-CH_2	1.7
Arg β-CH_2	1.8
Glu β-CH_2	2.0
GluNH β-CH_2	2.1
Thr CH_3	1.2

[a] The proton resonances listed are those from amino acyl residues possessing a potential hydrogen bonding group which also resonate in the region 0–2.2 ppm downfield of DSS according to the tabulation of McDonald and Phillips (1969) for random coil proteins.

Therefore, it seems likely that the proton groups effecting the H-2 NOE at 0.9 and 1.7 ppm belong to amino acid residues which have a functional group capable of forming hydrogen bonds with ADP or which have positively charged groups capable of interacting with the phosphoryl groups of ADP.

The frequency of the H_2 field which is most effective in promoting an NOE on the H-2 proton of ADP provides a clue to the identity of an amino acyl residue present at the active site of creatine kinase. Although many residues can be eliminated since H_2 is most effective at 0.9 and 1.7 ppm downfield of DSS, there are still several potential contributors to the NOE at these frequencies. As listed in Table III, there are five amino acid residues possessing either a potential hydrogen bonding function or a positively charged group, and also possessing a proton which resonates near 1.7 or 0.9 ppm.

The experiment with etheno-ADP, however, provides an argument against the NOE effectors being on a hydrogen-bond-donating amino acid residue. The etheno group on ADP blocks both the N-1 and the N-6 nitrogens of ADP from forming hydrogen bonds. Nevertheless, the H-2 proton of etheno-ADP in the binary enzyme complex still experiences a significant NOE with H_2 field applied at 0.9 or 1.7 ppm. It appears likely, therefore, that the NOE effectors are on an amino acid residue possessing a positively charged group. Table III indicates that a lysyl or an arginyl residue may be responsible.

The experiment with adenosine and creatine kinase supports this argument. Adenosine is a competitive inhibitor of the enzymatic reaction (Watts, 1973), and it lacks a phosphoryl group. In contrast to the nucleotide (see Table II), solutions with a concentration ratio of adenosine-to-enzyme binding site as low as 7.8 did not yield an NOE for the H-2 proton of the nucleoside. Although adenosine is not bound as tightly as the nucleotides to creatine kinase, its inhibitor constant is comparable to that of formate (Watts, 1973; Reed and

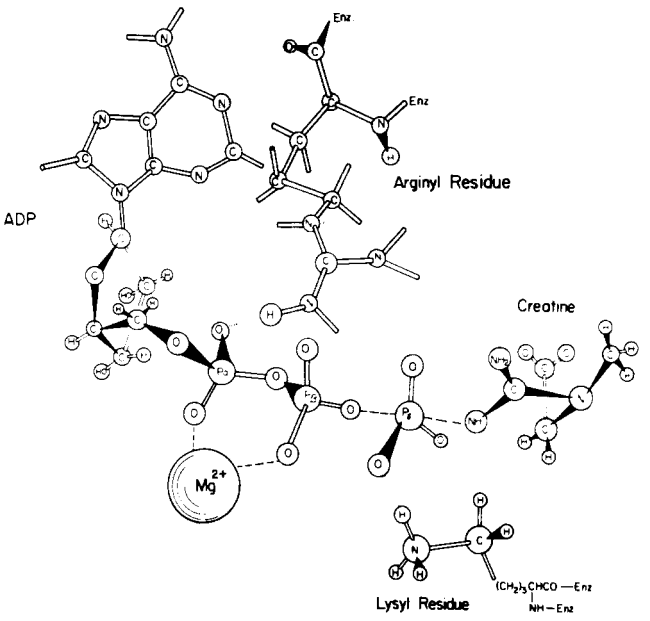

FIGURE 4: One possible schematic representation of the transition state complex formed in the creatine kinase catalyzed reaction. The postulated function of the essential arginine at the active site of the enzyme is illustrated.

McLaughlin, 1974). Yet formate exhibits a significant NOE in the enzyme-Mg(II)-ADP-creatine-formate complex at ligand-to-active site ratios as large as 64. These results imply that a phosphoryl group is necessary for the nucleotide H-2 proton to experience an NOE.

Comparison of the adenosine results with those of the nucleotides provided further support for the hypothesis that the enzyme protons effecting the ADP H-2 proton NOE are on an amino acid residue involved in binding the nucleotide via a phosphoryl group. As a competitive inhibitor, it would be assumed that the adenosine base is bound to the enzyme in a manner similar to the base in nucleotide substrates and inhibitors. With that reasonable assumption, it would be expected that the H-2 protons of adenosine and the nucleotides would experience similar NOE's if the NOE was caused by protons on a nearby residue which was not binding the nucleotide. The elimination of the NOE in the case of adenosine implied that the protons causing the NOE with the nucleotides are on the amino acid residue which binds the nucleotide phosphoryl group.

The lack of an observable H-2 proton NOE for ADP in the presence of creatine kinase with the essential arginyl residue modified (Borders and Riordan, 1975) suggests that the β- and γ-methylene protons of the arginyl residue may be responsible for the observed NOE's. A molecular model in which the positively charged guanidino group of an arginyl residue binds the α-phosphoryl of ADP indicates that such an interaction could lead ultimately to the observed nuclear Overhauser effects. In particular, the nucleotide conformation suggested by Hampton et al. (1972) and Schweizer et al. (1968) for AMP is feasible as the conformation of ADP in its complexes with creatine kinase. Binding of the guanidino to the α-phosphoryl rather than the β-phosphoryl was deemed more probable since AMP also gives rise to the H-2 proton NOE with creatine kinase (see Table II). Binding to both phosphoryl groups is also a possibility. A model of the postulated active site complex is given in Figure 4, illustrating the role of the guanidino group of an arginine residue binding the phosphoryl groups of ADP.

The work reported here illustrates the utility of nuclear Overhauser effect measurements for investigation of the detailed role of amino acyl residues in promoting enzymic catalysis. In conjunction with experiments involving pH change or chemical modification, it is possible to use NOE measurements to identify and deduce the catalytic function of amino acyl residues at the active site. Corollary information about which substrates, inhibitors, and activators promote conformational changes and how profound are these conformational changes may also be obtained via the intermolecular NOE experiments.

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Active-Site-Directed Inactivation of Aromatase from Human Placental Microsomes by Brominated Androgen Derivatives†

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ABSTRACT: Several brominated androgen derivatives were tested for their ability to inactivate microsomal aromatase from term human placenta. In the experimental protocol, the microsomal homogenate was incubated either with androstenedione or a brominated derivative of androstenedione (16α-bromo-6-ketoandrostenedione, 16α-bromoandrostenedione, 7α-(3′-bromoacetoxypropyl)androstenedione, 6α-bromoandrostenedione, or 6β-bromoandrostenedione) and reduced nicotinamide adenine dinucleotide phosphate in a nitrogen saturated buffer composed of glycerol, ethylenediaminetetraacetic acid, and dithiothreitol in tris(hydroxymethyl)aminomethane hydrochloride (pH 7.4) under nitrogen at 4 °C with shaking. After the incubation period, the microsomes were recovered by centrifugation and washed once before determining aromatase specific activity. The brominated androgen derivatives which inactivated aromatase were 7α-(3′-bromoacetoxypropyl)androstenedione and 6α-bromoandrostenedione. The structures of 6α- and 6β-bromoandrostenedione were unequivocally established by single crystal x-ray diffraction techniques. The extent of the enzyme inactivation by 6α-bromoandrostenedione was linearly proportional to the logarithm of its concentration. The evidence that this inactivation occurs at the aromatase active site is that androstenedione, when coincubated with 6α-bromoandrostenedione, protected aromatase from this inactivation. Progesterone provided much less protection than androstenedione. Furthermore, both 6α- and 6β-bromoandrostenedione are competitive inhibitors of androstenedione aromatization, as determined by a Lineweaver–Burk plot, and 6α-bromoandrostenedione gives the same type I cytochrome P-450 binding spectrum with placental microsomes as androstenedione. These data suggest that 6α-bromoandrostenedione is effective as an active-site-directed inhibitor of placental microsomal aromatase.

Estrogen synthetase, or aromatase, catalyzes the final step in the biosynthetic sequence from cholesterol to the estrogens. Studies of estrogen biosynthesis, utilizing the microsomal fraction of human term placenta (Ryan, 1959) as the source of the enzyme, have led to proposals that (1) androgen aromatization requires 3 mol of both molecular oxygen and NADPH and the participation of cytochrome P-450 (Thompson and Siiteri, 1974a, b), (2) androgens are converted directly to estrogens at a single active site without free intermediates (Hollander, 1962; Osawa and Shibata, 1973; Kelly et al., 1975), and (3) several different active sites are available to accommodate the various androgen substrates (Meigs and Ryan, 1971; Bellino and Osawa, 1974; Zachariah and Juchau, 1975; Canick and Ryan, 1975). Further definitive studies into the physical characteristics and macromolecular structure of aromatase have been limited by the instability of the enzyme and its refractoriness toward solubilization. To circumvent the problem of enzyme instability during solubilization and purification procedures, we searched for an affinity labeling reagent which, when obtained in a radioactive form, would permit the localization of the enzyme by radioactive detection rather than enzyme activity. This would also permit studies

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