Seeman, P. (1972) *Pharmacol. Rev. 24*, 583–655.
Shinoda, K., Yamaguchi, T., & Hori, R. (1961) *Bull. Chem. Soc. Jpn. 34*, 237–241.
Singleton, W. S., Gray, M. S., Brown, M. L., & White, J. L. (1965) *J. Am. Oil Chem. Soc. 42*, 53–56.
Skou, J. C. (1975) *Q. Rev. Biophys. 7*, 401–434.
Small, D. M. (1971) in *The Bile Acids* (Nair, P. B., & Kritchevsky, D., Eds.) Vol. 1, pp 249–356, Plenum Press, New York-London.
Stubbs, G. W., & Litman, B. J. (1978) *Biochemistry 17*, 220–225.
Swanson, P. D., Bradford, H. F., & McIlwain, H. (1964) *Biochem. J. 92*, 235–247.
Tanaka, R., & Strickland, K. P. (1965) *Arch. Biochem. Biophys. 111*, 583–592.
Tanford, C. (1970) *Adv. Protein Chem. 24*, 1–95.
Tanford, C., & Reynolds, J. A. (1976) *Biochim. Biophys. Acta 457*, 133–170.
Waggoner, A. S., Keith, A. D., & Griffith, O. H. (1968) *J. Phys. Chem. 72*, 4129–4132.
Wahler, B. E., & Wollenberger, A. (1958) *Biochem. Z. 329*, 508–520.
Wheeler, K. P., Walker, A. J., & Barker, D. M. (1975) *Biochem. J. 146*, 113–122.
Zaslavsky, B. Y., Ossipov, N. A., & Rogozhin, S. V. (1978) *Biochim. Biophys. Acta 510*, 151–159.

Creatine Kinase. Nuclear Magnetic Resonance and Fluorescence Evidence for Interaction of Adenosine 5′-Diphosphate with Aromatic Residue(s)†

Milan Vašák,* Kuniaki Nagayama, Kurt Wüthrich, Marina L. Mertens, and Jeremias H. R. Kägi

ABSTRACT: The structural features of the purine binding site of creatine kinase (CPK) were explored by ^{1}H NMR spectroscopy at 360 MHz, using the measurement of "truncated driven nuclear Overhauser effects" (TOE). Irradiation of the adenine C-2 and C-8 proton resonances in the CPK–ADP complex by this technique resulted in the negative enhancement of a number of resonances of the protein (intermolecular NOE's). Two of the affected resonances coincide with the irradiation frequencies shown previously to induce negative intermolecular NOE's in the adenine C-2 proton resonance of bound ADP [James, T. L. (1976) *Biochemistry 15*, 4724]. The occurrence of several NOE's in the aromatic region between 6.5 and 8.0 ppm is compatible with the location of one or more aromatic side chains near the adenine ring in the CPK–ADP complex. Independent evidence for an interaction between the purine moiety of the coenzyme and aromatic amino acid chromophores comes also from quenching studies of protein fluorescence. Binding of nucleoside phosphates reduces tryptophan emission of CPK. The extent of quenching by ADP and GDP corresponds to the relative magnitudes of the Förster overlap integrals, thus suggesting a resonance transfer mechanism. Since the calculated critical Förster distance for resonance transfer between ADP and the affected tryptophanyl residues in CPK is not larger than 5 Å, at least one tryptophanyl residue must be located in the immediate vicinity of the purine binding site of CPK. The data are in accordance with our previous proposal that the coenzyme-induced Cotton effects at 260 nm arise from a dipole–dipole interaction of the adenine transition with a nearby aromatic oscillator.

The structure of the binding sites of CPK[1] for ADP/ATP and creatine phosphate/creatine has been under investigation for a long time. Chemical modification studies carried out in the presence and absence of coenzymes and substrates have identified a number of amino acid residues located at or near the active center of CPK. Thus, the reaction with certain thiol reagents resulted in derivatization of a single active site cysteine (Mahowald et al., 1962) whose proximity to the substrate binding sites was demonstrated by magnetic resonance techniques using a covalently attached spin-label (Taylor et al., 1971; McLaughlin et al., 1976). The existence of one essential lysyl residue per subunit was deduced from the reaction with dansyl chloride (Kassab et al., 1968). Similarly, the involvement of arginine was inferred from the effect of butadiene or phenylglyoxal on the nucleotide binding properties of CPK (Borders & Riordan, 1975). Independent support for the location of both lysine and arginine in the active site was recently obtained also by James & Cohn (1974) and by James (1976), who observed a negative intermolecular NOE between resonances assigned to lysine and arginine and that of the ADP C-2 proton of the coenzyme in the ^{1}H NMR spectra of the enzyme–coenzyme complexes. An indication for an involvement of histidyl residues was provided by chemical modification with diethyl pyrocarbonate. Thus, Pradel & Kassab (1968) reported modification of one histidyl residue per subunit with concomitant complete loss of enzyme activity.

Except for a report that the inactivation of the enzyme by iodination is accompanied by the modification of two to three tyrosyl residues per subunit, no chemical evidence is available for the presence of aromatic amino acid residues in the active site of CPK (Fattoum et al., 1975). There are, however, data on record that the optical absorption of the aromatics in the enzyme is perturbed when the binary enzyme–coenzyme or

† From the Biochemisches Institut der Universität Zürich, CH-8028 Zürich, Switzerland (M.V., M.L.M., and J.H.R.K.), and the Institut für Molekularbiologie und Biophysik, Eigenössische Technische Hochschule, Hönggerberg, CH-8093 Zürich, Switzerland (K.N. and K.W.). *Received February 15, 1979.* This work was supported by Swiss National Science Foundation Grants 3.125-0.77 and 3.004.76.

[1] Abbreviations used: CPK, creatine kinase (adenosine 5′-triphosphate:creatine *N*-phosphotransferase, EC 2.7.3.2); NOE, nuclear Overhauser effect; TOE, truncated driven nuclear Overhauser effect; TSP, sodium 3-(trimethylsilyl)[2,2,3,3-$^2H_4$]propionate; CD, circular dichroism; ORD, optical rotatory dispersion.

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the ternary enzyme–coenzyme–substrate analogue complexes are formed. Thus, difference absorption spectra reflecting perturbation of tryptophanyl residues of the protein were observed on binding of ADP or ATP to CPK (Noda, 1963; Roustan et al., 1968). An interaction of the adenine moiety of the coenzymes with a vicinal tryptophanyl side chain was also proposed (Kägi et al., 1971) to be the basis of the large extrinsic Cotton effect of the CPK–coenzyme complexes. The magnitudes of the observed rotatory strength were in reasonable agreement with values obtained from theoretical calculations based on a model in which the stacked purine and indole rings interact by an electric dipole–dipole coupling mechanism.

The objective of the present work was to gain more direct spectroscopic evidence for the presence of aromatic residues in the ADP binding site of CPK. The approaches used were studies of intermolecular NOE in the 360-MHz ^{1}H NMR spectra of the CPK–ADP complex and the investigation of fluorescence resonance transfer between CPK and its ligands based on the Förster theory.

Materials and Methods

CPK of rabbit skeletal muscle (M_r 82 600) and sodium adenosine 5′-diphosphate were purchased from Boehringer; barium adenosine 5′-diphosphate was from Sigma Chemical Co.; deuterium oxide (98%), DCl, and NaOD were products of Fluka. The 99.91% deuterium oxide was a product of Stohler Isotope Chemicals.

For fluorescence titration studies, barium adenosine 5′-diphosphate was converted to the free acid by mixing approximately 500 μmol with 4 mL of cation-exchange resin (Bio-Rad 50 W-X2; 200–400 mesh) in the hydrogen form. After filtration through a 0.2-μm pore size cellulose nitrate membrane (Sartorius), the free acid was neutralized with NaOH to pH 8. The purity of ADP was monitored by thin-layer chromatography on poly(ethylenimine)–cellulose (Macherey-Nagel & Co.) using 1.3 M LiCl (Merck) as a solvent (Randerath & Randerath, 1967).

The concentration of CPK was determined spectrophotometrically at 280 nm by using a specific absorbancy index of 0.88 mL/(mg cm) (Kuby et al., 1954), and that of ADP was determined by using a millimolar extinction coefficient ϵ = 15.4 (Pabst Laboratories, 1956). The enzymatic activity was monitored by using a commercial kit (Boehringer). No significant changes in activity occurred in lyophilization and during NMR measurements. Samples for NMR spectroscopy were prepared by first lyophilizing the enzyme and sodium adenosine 5′-diphosphate respectively 3 times from 98% D_2O, pD 7.6, and then dissolving the lyophilized substance in 99.91% D_2O. The pD values correspond to the pH meter readings without correction for isotope effects (Kalinichenko, 1976). The protein concentration used for the NMR studies was 60 mg/mL. The concentration of ADP was between 3 and 6 mM, corresponding to more than 95% saturation of the enzyme binding sites ($K_D = 1.6 \times 10^{-4}$ M) (see Results). High-resolution Fourier transform ^{1}H NMR spectra were recorded at 24 °C on a Bruker HXS-360 spectrometer.

For the NOE studies of CPK and the CPK–ADP complex, 6000 transients with a 6-s repetition rate were accumulated. For the CPK–MgADP complex, 3000 transients with a 6-s repetition rate were used. All experiments were carried out on independent samples 3 times. Chemical shifts are quoted in parts per million (ppm) downfield from sodium 3-(trimethylsilyl)[2,2,3,3-$^2H_4$]propionate (TSP).

Structural information on the complexes of CPK with ADP and Mg^{2+} was obtained from studies of NOE's in the ^{1}H NMR spectra. NOE's were measured in NOE difference spectra (Richarz & Wüthrich, 1978). In order to obtain specific NOE's in spite of spin diffusion which in this high molecular weight system leads effectively to saturation transfer over long distances (Kalk & Berendsen, 1976), we recorded TOE's (Dubs et al., 1979; Wagner & Wüthrich, 1979). In TOE experiments the presaturation field is applied for a relatively short time so that the steady-state situation is not reached and the influence of spin diffusion, as compared to direct interactions between nearby nuclei, is reduced.

The fluorescence measurements were carried out at 30 ± 0.1 °C in a 1-cm quartz fluorescence cuvette in a Hitachi Perkin-Elmer MPF-2A spectrofluorometer equipped with a 150-W xenon lamp. The fluorescence data were corrected as described elsewhere (Mertens & Kägi, 1979).

Calculations of the critical distance, R_0, at which tryptophanyl fluorescence of CPK is quenched 50% by ADP were made from the Förster equation (Förster, 1948, 1965)

$$R_0 = 9700(JK^2Q_0n^{-4})^{1/6}\ \text{Å} \qquad (1)$$

where for the dipole–dipole orientation factor the random average value $K^2 = 2/3$ was employed,[2] for the refractive index the value of $n = 1.0$ was employed (assuming resonance transfer between contiguous groups (Guéron et al., 1967; see Discussion)), and for the quantum yield of the donor (tryptophanyl residues in CPK) in the absence of the quenching ligands the value $Q_0 = 0.11$ was employed (Burshtein et al., 1973). The spectral overlap integral J (in centimeters^3 moles^{-1} liter) was determined from the fluorescence emission spectrum of the protein and from the absorption spectrum of ADP by using the expression

$$J = \frac{\int F(\lambda)\epsilon(\lambda)\lambda^4\,d\lambda}{\int F(\lambda)\,d\lambda} \qquad (2)$$

where $\epsilon(\lambda)$ is the molar extinction coefficient of the acceptor in units of centimeters^{-1} moles^{-1} liter and $F(\lambda)$ is the donor fluorescence in arbitrary units.

Results

A normal Fourier transform ^{1}H NMR spectrum of CPK and the TOE difference spectra obtained with preirradiation at 8.18 ppm (ADP C-2 proton resonance) for CPK and its binary complex with ADP are shown in Figure 1. The driven NOE experiments were truncated after 1 s. For these conditions one anticipates that the TOE difference spectra are affected by the rapidly migrating spin diffusion through the rigid lattice in the protein (Akasaka et al., 1978; Wagner & Wüthrich, 1979). This is borne out by the appearance of numerous broad resonances in the spectra b and c in Figure 1. For the structural interpretation of the spectral features of the ADP complex given in this report, we make the assumption that the NOE's arising from irradiation of resonances of the protein at 8.18 ppm are the same in CPK and in the CPK–ADP complex.[3] Additional lines would then be expected in the TOE difference spectrum of the CPK–ADP

[2] The choice of $K^2 = 2/3$ implies a random motion of at least one of the chromophores (Dale & Eisinger, 1975). Inasmuch as this condition is not met in the system investigated, K^2 and, hence, R_0 could have a different value. However, with 80% probability, the critical distance will at most differ by ±30% or even less if the energy acceptor has some rotational freedom and the anisotropy of the donor and acceptor transitions is not extreme (Stryer, 1978).

[3] The NMR resonances of free ADP and of ADP bound to CPK are not resolved. Thus, the intensity of the monitored nuclei is affected by the NOE exerted both by the bound ADP and by the cross-saturation effect from free ADP through the exchange process (Redfield & Gupta, 1971).

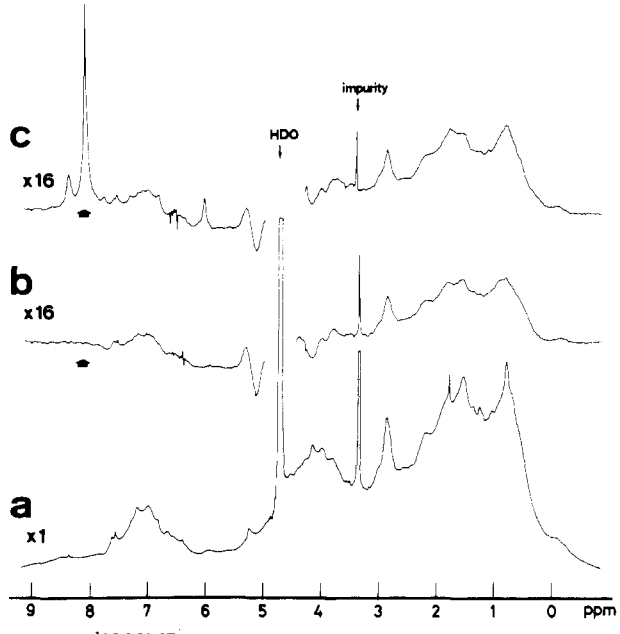

FIGURE 1: ^{1}H NMR spectra (360 MHz) of CPK and the CPK–ADP complex. The CPK concentration was 60 mg/mL, ADP concentration was 4.1 mM, pD 7.6, and T = 24 °C. (a) Normal Fourier transform spectrum of CPK. (b) TOE difference spectrum of CPK recorded with a preirradiation time of 1 s. (c) TOE difference spectrum of the CPK–ADP complex recorded with a preirradiation time of 1 s. The irradiation frequency at 8.18 ppm is indicated by the arrow.[3] The numbers at (b) and (c) reflect the ordinate expansion factors with respect to (a).

complex to arise from the preirradiation of the C-2 proton of ADP at 8.18 ppm, where the most intense resonances would come from protons located near the preirradiated ADP C-2 proton in the protein–ADP complex. Comparison of the spectra b and c in Figure 1 indicates that there are indeed some additional resonances in the difference spectrum c. To pick up these specific effects arising from ADP binding, we subtracted the TOE difference spectrum obtained for CPK from that of the CPK–ADP complex or the CPK–MgADP complex. Figures 2 and 3 show such differences of TOE difference spectra (d and e) together with the normal Fourier transform spectra of CPK (a) and the CPK–ADP complex (b) and the ordinary difference spectrum (c) obtained by subtracting the spectrum a from spectrum b. The feature of spectra d and e indicates only those NOE's which arise from interactions between ADP and the protein. In the aliphatic region (Figure 2), the most prominent NOE's displayed in spectra d and e are associated with resonances at about 1.78 and 0.83 ppm which correspond to the irradiation frequences that were shown previously to induce negative intermolecular NOE's in the adenine C-2 proton resonance of CPK-bound ADP (James, 1976) and which were attributed to the β- and γ-methylene protons of an arginyl residue of the enzyme. In addition, there are also intermolecular NOE's discernible at about 2.98 and 0.34 ppm.

Under the above conditions, TOE difference spectra of CPK and its binary complex with ADP were also obtained with preirradiation at 8.45 ppm (ADP C-8 proton resonance). The aliphatic region revealed three NOE's at about 0.83, 1.78, and 2.98 ppm (Table II). The latter NOE peak exhibits the largest enhancement factor. The other two NOE peaks coincide in position with the resonances obtained by irradiation of the C-2 ADP proton. The spatial separation of the C-2 and C-8 protons could account for both the differences in the enhancement factors (Table I and Table II) and the absence of the 0.34-ppm resonance (Figure 3) in the C-8 irradiation experiment.

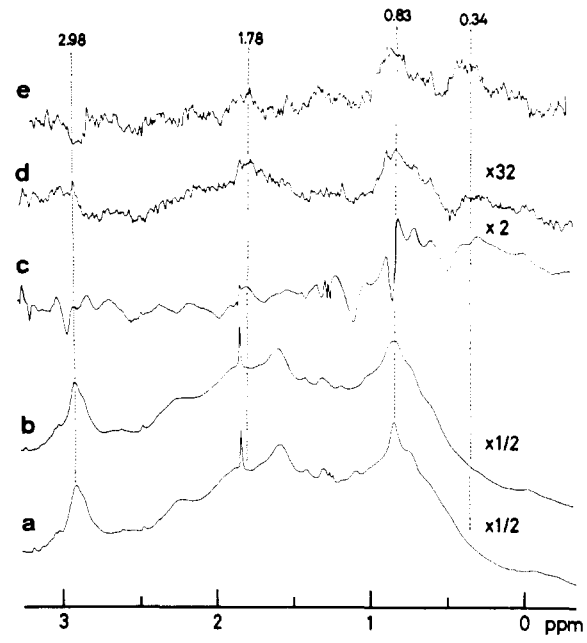

FIGURE 2: Aliphatic region of the 360-MHz ^{1}H NMR spectra of CPK, the CPK–ADP complex, and the CPK–MgADP complex. The CPK concentration was 60 mg/mL, ADP concentration was 4.1 mM, pD 7.6, and T = 24 °C; in experiment e the solution contained in addition 20 mM $MgCl_2$. For the spectra a–d, 6000 transients were accumulated; for spectrum e, 3000 transients were accumulated. (a) Normal Fourier transform spectrum of CPK. (b) Normal Fourier transform spectrum of the CPK–ADP complex. (c) Difference spectrum (b – a). (d) Difference between the TOE difference spectra obtained with preirradiation at 8.18 ppm for the CPK–ADP complex and CPK (= spectrum c – spectrum b of Figure 1). (e) Difference between the TOE difference spectra (not shown) obtained with preirradiation at 8.18 ppm for the CPK–MgADP complex and CPK. The numbers at the spectra b–d reflect the ordinate expansion factors with respect to spectrum a in this figure.

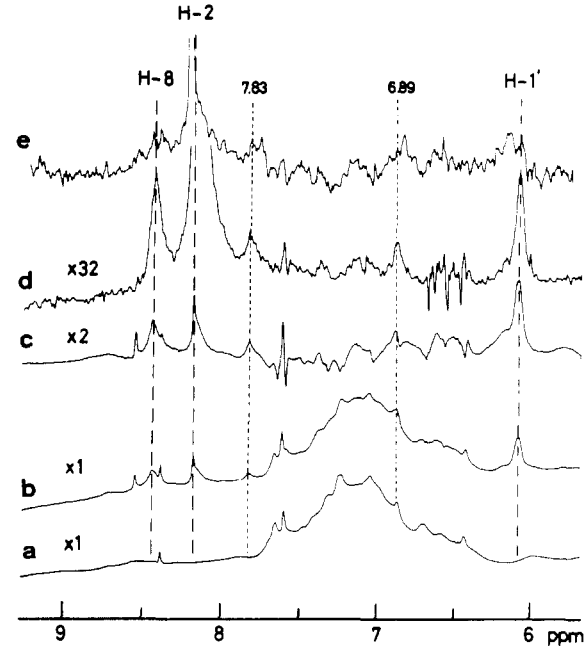

FIGURE 3: Aromatic region of the 360-MHz ^{1}H NMR spectra of CPK, the CPK–ADP complex, and the CPK–MgADP complex. For experimental conditions and symbols see the legend of Figure 2. The numbers at the spectra a–d reflect the ordinate expansion factors with respect to spectrum a in Figure 2.

In the aromatic region (Figure 3), irradiation of the ADP C-2 proton causes the appearance of two strong NOE's for other protons of the coenzyme. The resonance at about 8.45

Table I: Negative Enhancements of Creatine Kinase and ADP Resonances Obtained with Preirradiation at 8.18 ppm (ADP C-2 Proton Resonance)

chemical shift (ppm)	assignment	enhancement (%)
0.34		~10[c]
0.83	Arg[a]	~10[c]
1.78	Arg[a]	~10[c]
2.98		<10[c]
6.09	C-1′ proton of (ADP) ribose[b]	~10[c]
6.89	Trp, Tyr, or Phe	~10[c]
7.83	Trp, Tyr, or Phe	15
8.18	C-2 proton of (ADP) adenine[b]	*d*
8.45	C-8 proton of (ADP) adenine[b]	20

[a] From James (1976). [b] From Chan et al. (1964) and Broom et al. (1967). [c] Estimation is made relative to the intensity of the corresponding signal in the ordinary difference spectrum c in Figures 2 and 3. [d] Irradiated position.

Table II: Negative Enhancements of Creatine Kinase and ADP Resonances Obtained with Preirradiation at 8.45 ppm (ADP C-8 Proton Resonance)

chemical shift (ppm)	assignment	enhancement (%)
0.83		<10[b]
1.78		<10[b]
2.98		~10[b]
6.09	C-1′ proton of (ADP) ribose[a]	<10[b]
7.26	Trp, Tyr, or Phe	~10[b]
7.02	Trp, Tyr, or Phe	~10[b]
7.83	Trp, Tyr, or Phe	20
8.18	C-2 proton of (ADP) adenine[a]	20
8.45	C-8 proton of (ADP) adenine[a]	*c*

[a] From Chan et al. (1964) and Broom et al. (1967). [b] Estimation is made relative to the intensity of the corresponding signal in the ordinary difference spectrum. [c] Irradiated position.

ppm belongs to the C-8 proton of the adenine moiety (Chan et al., 1964) while that at about 6.09 ppm corresponds to the C-1′ proton of the ribose moiety (Broom et al., 1967). In addition, the spectra d and e show clearly that there are at least two NOE's (6.89 and 7.83 ppm) which most likely correspond to aromatic resonances of the protein.

The irradiation of the C-8 ADP proton gave rise to a total of five negative NOE's in the aromatic region (Table II). The two resonances at about 6.09 and 8.18 ppm belong to the C-1′ proton and the C-2 proton of the ribose and adenine moieties of ADP, respectively (Chan et al., 1964; Broom et al., 1967). The three remaining NOE's correspond to aromatic resonances of the protein. The signal at 7.83 ppm is probably identical with the NOE-affected resonance observed upon irradiation of the C-2 ADP proton. In addition, two different NOE's at 7.26 and 7.02 ppm are discernible. On the other hand, the NOE observed at 6.89 ppm upon C-2 proton irradiation (Figure 3) was not noticeable in this experiment.

In spite of the lower spectral resolution obtained with the CPK–MgADP complex (Figures 2 and 3), there are, especially in the aliphatic region, a number of differences from the CPK–ADP complex. Thus, in the complex containing Mg^{2+}, there is an increase in the resonance intensity at 0.34 ppm and a shift of the resonances which in the absence of Mg^{2+} are located at 2.98 ppm. Because of the lower signal to noise ratio, deductions about the aromatic region of the protein are less certain. However, there seems to be a diminution of the intensities of the NOE's observed for the adenine C-8 and the ribose C-1′ proton resonances in the CPK–MgADP complex as compared to those of the complex without Mg^{2+}.

The chemical shift and the change in intensity for the observed enhancements are listed in Tables I and II. The most reliable values were obtained for the 6.09-ppm resonance and for the 8.45- and 8.18-ppm adenine resonances and for the aromatic resonance of the protein at 7.83 ppm which are located outside of the bulk of the protein signals.

Information about the vicinity of the coenzyme binding site comes also from a study of ADP-binding on the fluorescence properties of CPK. Figure 4 shows the fluorescence spectrum of the enzyme which by its shape and location can be attributed to tryptophanyl residues. On complex formation with ADP, the fluorescence is partially quenched, and the maximum is shifted from 331 nm to approximately 328 nm. The fluorescence characteristics of the quenched tryptophanyl residue(s) displayed by the difference fluorescence spectrum (Figure 4, below) show a maximum at 340 nm. The extent of fluorescence quenching depends on the degree of occupation of the binding sites by ADP and reaches maximally 14% of the total

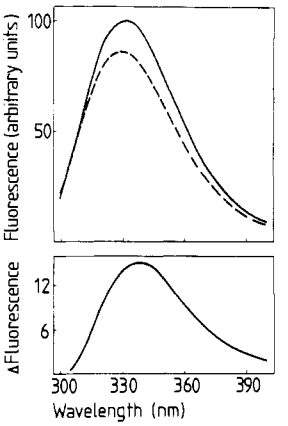

FIGURE 4: Effect of ADP binding on fluorescence emission spectrum of CPK. Above: (—) CPK, 0.1 mg/mL, in 0.05 M Tris–0.03 M acetic acid buffer, pH 8.0; excitation was at 290 nm; (---) CPK, 0.1 mg/mL, and ADP, 6 mM, in 0.05 M Tris–0.03 M acetic acid buffer, pH 8.0; excitation was at 290 nm. Below: Fluorescence emission difference spectrum of CPK vs. CPK–ADP (data from spectra of Figure 4 above).

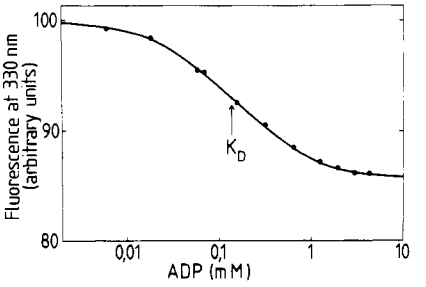

FIGURE 5: Fluorescence quenching titration of CPK with ADP. Conditions: same as described in the legend of Figure 4; emission was at 330 nm.

fluorescence. The dissociation constant of the CPK–ADP complex determined by fluorescence quenching titration is 1.6×10^{-4} M (Figure 5). This value is identical with that of the CPK–MgADP complex obtained either from fluorescence quenching titration (Mertens, 1978) or from kinetic studies (Morrison & James, 1965).

Tryptophan fluorescence is also quenched by binding of IDP (12%) and of GDP (24%) to CPK. The large effect of GDP corresponds to the much larger spectral overlap of its absorption red edge with the protein fluorescence spectrum, suggesting a Förster energy transfer mechanism to be operative (Förster, 1948, 1965). The critical Förster transfer distances R_0 for quenching of tryptophan fluorescence by ADP and GDP as calculated from the overlap integrals J of the donor and acceptor spectra ($J_{Trp/ADP} = 1.4 \times 10^{-19}$ cm^3 M^{-1}; $J_{Trp/GDP}$ =

4.5×10^{-18} cm^3 M^{-1}) were 4.5 and 8 Å, respectively.

Discussion

The ^{1}H NMR experiments described in this paper document that presaturation of the C-2 and C-8 protons of ADP produces NOE's on some resonances of CPK. Thus, this study complements the earlier NOE measurements by James (1976), who observed NOE's on the ADP C-2 proton resonance in the CPK–ADP complex when certain resonances of the protein were subjected to irradiation with a strong radio-frequency field.

Effects of ADP binding are also manifested in the normal ^{1}H NMR difference spectrum without NOE (spectrum c in Figures 2 and 3), some of which are coinciding with the location of resonances affected by the NOE. Others, such as the 8.6-ppm low-field difference peak, which from its chemical shift might be attributed to C-2 protons of histidine (Wüthrich, 1976; Dwek, 1973), do not show a NOE. Such features probably arise from indirect effects of coenzyme binding on protein conformation. Small changes of the conformation of CPK accompanying binding of ADP have been demonstrated in a number of previous studies (Lui & Cunningham, 1966; Hammes & Hurst, 1969; McLaughlin, 1974).

The most prominent aliphatic peaks located at 0.83 and 1.78 ppm in the difference of the TOE difference spectra (spectrum d in Figure 2) coincide with the positions where irradiation caused NOE's on the C-2 proton resonance of ADP (James, 1976). The remaining four peaks noticeable in the spectrum d of Figures 2 and 3 apparently escaped detection in the earlier experiments. From their chemical shifts, the NOE signals at 6.89 and 7.83 ppm (spectrum d in Figure 3), when the preirradiation pulse to the C-2 ADP proton was employed, and those at 7.83, 7.26, and 7.02 ppm (Table II), when the preirradiation pulse to the C-8 ADP proton was employed, are to be attributed to protons of aromatic side chains. This indicates that, in addition to the previously evidenced occurrence of aliphatic protons near the binding site of ADP (James, 1976), one or several aromatic residues of CPK must be at a comparable distance from the ADP in the complex. The conclusion that these NOE's reflect a specific close-range interaction of the C-2 and C-8 protons of adenine with vicinal aromatic amino acid residues is also supported by the observation that preirradiation at the two resonance frequencies produces NOE patterns that differ considerably from each other both in the aliphatic and in the aromatic region (Tables I and II). This specificity in the magnetization transfer reflects most likely the different microenvironment at the two protons.

The effects of the presence of Mg^{2+} on the NOE's observed in spectrum e as compared to those seen in spectrum d in Figures 2 and 3 are presumably attributable to chelation of Mg^{2+} by the pyrophosphate group of ADP and may indicate a metal ion induced change in the geometry of the enzyme–coenzyme complex. Evidence for such a metal ion dependent effect was recently obtained also from a comparative circular dichroism study of the same complexes (Mertens, 1978).

Independent evidence for the occurrence of aromatic amino acid residues at the coenzyme binding site comes from the observation that binding of adenosine, inosine, and guanosine phosphates to CPK quenches protein tryptophan fluorescence (Figure 4). The different extent of fluorescence quenching observed in the various complexes is in accordance with the different size of the spectral overlap of the donor (tryptophan) and acceptor (purine) chromophores and implies that quenching proceeds by a classical Förster resonance transfer mechanism. The efficiency of quenching by this dipole–dipole coupling process depends on the reciprocal of the sixth power of the distance R between chromophores and on the critical Förster transfer distance R_0 for which transfer (= quenching) is 50% and which is a function of the spectroscopic and geometric features of the complex (see Materials and Methods). Due to the very small overlap integral J of the absorption spectrum of ADP and the tryptophanyl emission spectrum of CPK, the critical Förster distance for the tryptophan–ADP pair is very small (4.5 Å). The corresponding value for the tryptophanyl–GDP pair is larger (8 Å) and thus accounts for the more pronounced quenching of CPK fluorescence by this coenzyme. It is of interest also that the magnitude of the calculated transfer distances is comparable to the values obtained by Guéron et al. (1967) for resonance energy transfer between pairs of nucleotides. While it is admitted that at distances below 10 Å such distance calculations are affected by some uncertainty, it is clear from these results that quenching by the bound ADP can occur only if one or more tryptophanyl residues are located in the immediate vicinity of the coenzyme binding site.

A further indication that the affected tryptophanyl side chain is situated at the protein surface comes from the spectral position of its emission band (Figure 4). The fact that the difference fluorescence maximum is displaced toward the long-wavelength side of the fluorescence maximum of the protein (330 nm) indicates a relatively more polar environment of this residue. According to Burshtein et al. (1973), an emission maximum of 340 nm is characteristic of protein tryptophanyl residues which are partially exposed to water and, hence, located at the surface of the protein. Support for this inference comes also from perturbation difference absorption spectroscopic studies of Fattoum et al. (1975), who found that two of the four tryptophanyl residues are exposed.

The presence of one or more aromatic residues at the coenzyme binding site is compatible with the hydrophobic character of the purine binding pocket that was reported for a number of enzymes (Rossmann et al., 1974). That this residue is most likely one of the four tryptophans of CPK is suggested not only by the present fluorescence data but also by our earlier demonstration that the ORD and CD features of the complexes of CPK with adenine coenzymes can be generated by dipole–dipole coupling between the adenine chromophore and a nearby tryptophanyl side chain (Kägi et al., 1971; Mertens, 1978). The induction of rotatory power depends critically on the close proximity of the interacting groups. Taken together, our earlier and the present spectroscopic studies afford thus internally consistent evidence for the postulated occurrence of an aromatic residue in the purine binding region of CPK.

References

Akasaka, K., Konrad, M., & Goody, R. S. (1978) *FEBS Lett. 96*, 287.

Borders, C. L., Jr., & Riordan, J. F. (1975) *Biochemistry 14*, 4699.

Broom, A. D., Schweizer, M. P., & Ts'o, P. O. P. (1967) *J. Am. Chem. Soc. 89*, 3612.

Burshtein, E. A., Vedenkina, N. S., & Ivkova, M. N. (1973) *Photochem. Photobiol. 18*, 263.

Chan, S. I., Schweizer, M. P., Ts'o, P. O. P., & Helmkamp, G. K. (1964) *J. Am. Chem. Soc. 86*, 4182.

Dale, R. E., & Eisinger, J. (1975) in *Biochemical Fluorescence: Concepts* (Chen, R. F., & Edelhoch, M., Eds.) Vol. 1, p 115, Marcel Dekker, New York.

Dubs, A., Wagner, G., & Wüthrich, K. (1979) *Biochim. Biophys. Acta 577*, 177.

Dwek, R. A. (1973) *Nuclear Magnetic Resonance in Biochemistry*, Clarendon Press, Oxford.

Fattoum, A., Kassab, R., & Pradel, L. A. (1975) *Biochim. Biophys. Acta 405*, 324.
Förster, T. (1948) *Ann. Phys. (Leipzig) 2*, 55.
Förster, T. (1965) in *Modern Quantum Chemistry* (Sinanoglu, O., Ed.) Part III, p 93, Academic Press, New York.
Guêron, M., Eisinger, J., & Shulman, R. G. (1967) *J. Chem. Phys. 47*, 4077.
Hammes, G. G., & Hurst, J. K. (1969) *Biochemistry 8*, 1083.
James, T. L. (1976) *Biochemistry 15*, 4724.
James, T. L., & Cohn, M. (1974) *J. Biol. Chem. 249*, 2599.
Kägi, J. H. R., Li, T. R., & Vallee, B. (1971) *Biochemistry 10*, 1007.
Kalinichenko, P. (1976) *Stud. Biophys. 58*, 235.
Kalk, A., & Berendsen, H. J. C. (1976) *J. Magn. Reson. 24*, 343.
Kassab, R., Roustan, C., & Pradel, L. A. (1968) *Biochim. Biophys. Acta 167*, 308.
Kuby, S. A., Noda, L., & Lardy, H. A. (1954) *J. Biol. Chem. 209*, 191.
Lui, N. S. T., & Cunningham, L. (1966) *Biochemistry 5*, 114.
Mahowald, T. A., Noltmann, E. A., & Kuby, S. A. (1962) *J. Biol. Chem. 237*, 1535.
McLaughlin, A. C. (1974) *J. Biol. Chem. 249, 1445.*
McLaughlin, A. C., Leigh, J. S., Jr., & Cohn, M. (1976) *J. Biol. Chem. 251*, 2777.
Mertens, M. L. (1978) Ph.D. Thesis, University of Zurich.
Mertens, M. L., & Kägi, J. H. R. (1979) *Anal. Biochem. 96*, 448.
Morrison, J. F., & James, E. (1965) *J. Biol. Chem. 241*, 4758.
Noda, L. (1963) *Abstracts of Papers*, 143rd National Meeting of the American Chemical Society, Cincinnati, OH, p 19A.
Pabst Laboratories (Milwaukee, WI) (1956) *Circular OR-10.*
Pradel, L. A., & Kassab, R. (1968) *Biochim. Biophys. Acta 167*, 317.
Randerath, K., & Randerath, E. (1967) *Methods Enzymol. 12*, 323–347.
Redfield, A. G., & Gupta, R. K. (1971) *Cold Spring Harbor Symp. Quant. Biol. 36*, 405.
Richarz, R., & Wüthrich, K. (1978) *J. Magn. Reson. 30*, 147.
Rossmann, M. G., Moras, D., & Olsen, K. W. (1974) *Nature (London) 250*, 194.
Roustan, C., Kassab, R., Pradel, L. A., & Thoai, N. V. (1968) *Biochim. Biophys. Acta 167*, 326.
Stryer, L. (1978) *Annu. Rev. Biochem. 47*, 822.
Taylor, J. S., McLaughlin, A. C., & Cohn, M. (1971) *J. Biol. Chem. 246*, 6029.
Wagner, G., & Wüthrich, K. (1979) *J. Magn. Reson. 33*, 675.
Wüthrich, K. (1976) *NMR in Biological Research: Peptides and Proteins*, North-Holland/American Elsevier, Amsterdam.

Subunit Association and Side-Chain Reactivities of Bovine Erythrocyte Superoxide Dismutase in Denaturing Solvents†

Douglas P. Malinowski and Irwin Fridovich*

ABSTRACT: The copper- and zinc-containing superoxide dismutase of bovine erythrocytes retains its native molecular weight of 32 000 in 8.0 M urea for at least 72 h at 25 °C, as evidenced by sedimentation equilibrium analysis. Subsequent to prolonged exposure to urea, the dimeric enzyme could be dissociated by sodium dodecyl sulfate in the absence of reductants, indicating the absence of unnatural disulfide cross-links. The sulfhydryl group of cysteine-6 was unreactive toward 5,5′-dithiobis(2-nitrobenzoic acid) or bromoacetic acid in both neutral buffer and 8.0 M urea. The histidine residues of the enzyme were resistant to carboxymethylation in neutral buffer and 8.0 M urea. However, when the enzyme was exposed to bromoacetic acid in the presence of 6.0 M guanidinium chloride and 1 mM (ethylenedinitrilo)tetraacetic acid (EDTA), both sulfhydryl and histidine alkylation were observed. Guanidinium chloride (6.0 M) increased the reactivity of the sulfhydryl group of cysteine-6 and allowed the oxidative formation of disulfide-bridged dimers. This was prevented by 1 mM EDTA. It follows that 8.0 M urea neither dissociates the native enzyme into subunits nor produces a conformation detectably different than that possessed under native conditions.

The copper- and zinc-containing superoxide dismutase from bovine erythrocytes (BESOD)[1] is a homodimer of molecular weight 32 000 (Evans et al., 1974; Steinman et al., 1974; Abernethy et al., 1974). X-ray diffraction analysis has allowed elucidation of the structure and identification of the ligands at the active site of this enzyme (Richardson et al., 1975a,b). The copper is held within the active site through the imidazole rings of histidine-44, -46, -61, and -118, arranged in a distorted square-planar configuration. The copper participates in the catalytic process through a Cu(II)/Cu(I) redox cycle (Klug et al., 1972; Klug-Roth et al., 1973; Rotilio et al., 1972). The zinc, which is 6 Å away from the copper, is liganded by histidine-61, -67, and -78 and by aspartate-81 in a tetrahedral arrangement. The zinc appears to serve a secondary structural role (Forman & Fridovich, 1973; Lippard et al., 1977).

The enzyme is unusually stable and is unchanged by an isolation procedure which utilizes organic solvents (McCord & Fridovich, 1969). Its catalytic activity is unaffected by 8.0 M urea or by 4% sodium dodecyl sulfate ($NaDodSO_4$) and

† From the Department of Biochemistry, Duke University Medical Center, Durham, North Carolina 27710. *Received June 11, 1979.* This work was supported by Research Grants GM-10287 and ESO-1908 from the National Institutes of Health, Bethesda, MD, and DAAG29-78-G-0088 from the U.S. Army Research Office, Research Triangle Park, NC, and grants from Merck Sharp & Dohme Research Laboratories, Rahway, NJ.

[1] Abbreviations used: EDTA, (ethylenedinitrilo)tetraacetic acid; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); BESOD, bovine erythrocyte superoxide dismutase; $NaDodSO_4$, sodium dodecyl sulfate.

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