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Tryptophan Residues of Creatine Kinase: A Fluorescence Study†

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ABSTRACT: Spectroscopic studies of rabbit skeletal muscle creatine kinase (CPK) and its complexes with adenosine phosphates have long suggested the occurrence of a tryptophan residue at or near the coenzyme binding sites [Kägi, J. H. R., Li, T.-K., & Vallee, B. L. (1971) *Biochemistry 10*, 1007-1015; Price, N. C. (1972) *FEBS Lett. 24*, 21-23]. This conjecture was further supported by nuclear Overhauser effect (NOE) ^{1}H NMR studies indicating through-space interactions between protons of the adenine ring of bound ADP and one or more aromatic side chains of the proteins [Vašák, M., Nagayama, K., Wüthrich, K., Mertens, M. L., & Kägi, J. H. R. (1979) *Biochemistry 18*, 5050-5055]. Further evidence for a tryptophan residue in the environment of the active site has now been obtained by fluorescence-quenching studies using iodide and acrylamide as external quenchers. Thus, while by the addition of iodide the tryptophan fluorescence of unliganded CPK is reduced to about 75% of the unquenched control, no such effect is manifested upon addition of this quencher to the CPK·ADP and CPK·ATP complexes. Similarly, the relative effectiveness of quenching of the CPK–coenzyme complexes by acrylamide is only about 60% of that measured in the unliganded enzyme. Both these data and the spectral characteristics of the quenched fluorescence suggest that coenzyme binding perturbs a tryptophan residue that is close to the active site and that is partially exposed to the solvent. The differential effectiveness of external quenchers on unliganded and liganded CPK allows the determination of the ligand binding equilibria by fluorescence-quenchability titration. The values obtained for complexes of CPK with ATP, ADP, and AMP are in good agreement with those obtained from other measurements.

Although the spatial structure of creatine kinase (CPK)[1] is still unknown, much information has been collected over the past 25 years on the organization of the binding sites of its substrates ADP/ATP and creatine phosphate/creatine. By chemical and physical studies carried out in the presence and absence of coenzymes and substrates, a number of amino acid residues have been identified 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 site was demonstrated by magnetic resonance techniques using a covalently attached spin-label (Taylor et al., 1971; McLaughlin et al., 1976). From analogous chemical modification studies, the presence of lysine (Kassab et al., 1968), arginine (Borders & Riordan, 1975), and histidine (Pradel & Kassab, 1968) at the active site was also inferred.

There is also some evidence for the occurrence of aromatic amino acids, especially tryptophan, at the active center. 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). This interaction was proposed to be the basis of the large extrinsic Cotton effect of the CPK–coenzyme complexes (Kägi et al., 1971). The presence of a tryptophan near the coenzyme binding site was also suggested by the observation that a part of the tryptophan fluorescence of CPK is quenched on coenzyme binding (Price, 1972; Mertens, 1978) and is consistent with ^{1}H NMR data, which, on the basis of measurements of "truncated driven nuclear Overhauser effects", indicated that the adenine ring of the coenzymes is close to the location of

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[1] Abbreviations: CPK, creatine kinase (adenosine-5′-triphosphate: creatine *N*-phosphotransferase, EC 2.7.3.2); Gdn·HCl, guanidine hydrochloride.

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one or more aromatic side chains of the enzyme (Vašák et al., 1979).

The objective of the present work was to substantiate further the occurrence of a tryptophan residue at or near the coenzyme binding site of CPK. In the present study, the quenching of tryptophan fluorescence by the quenching agents iodide and acrylamide both in CPK and in its complexes with ADP, ATP, and AMP was examined. The data provide evidence that CPK possesses a tryptophan residue that is selectively quenchable by iodide and whose quenchability is abrogated completely upon binding of ADP and ATP.

Experimental Procedures

Materials. CPK of rabbit skeletal muscle, lactate dehydrogenase of rabbit muscle, pyruvate kinase, creatine, phosphoenolpyruvate, NADH, disodium adenosine 5′-mono- and 5′-triphosphates were purchased from Boehringer; sodium adenosine 5′-diphosphate, L-tryptophan, and Trizma base were from Sigma Chemical Co; acrylamide was a product of Fluka. KI was a product of Merck. All aqueous solutions were prepared with water that had been redistilled. Guanidine hydrochloride (Gdn·HCl) was obtained from Pierce. CPK and L-tryptophan were prepared freshly in Tris–acetic acid buffer of pH 8 containing 0.05 M Trizma base. Nucleoside mono-, di-, and triphosphates were dissolved in water and neutralized with NaOH to pH 8. Solutions of acrylamide, KI, NaCl, and Gdn·HCl were also prepared in water. Stock solutions of CPK (3.3 mg mL^{-1}) were kept at 4 °C and were used within a few days of preparation.

Methods. Absorbance measurements were made with a Cary 15 recording spectrophotometer. The concentration of CPK was determined spectrophotometrically at 280 nm by using a specific absorbancy index of 0.88 mL mg^{-1} cm^{-1} (Kuby et al., 1954), and those of AMP, ADP, and ATP were determined by using a millimolar extinction coefficient $\epsilon_{259,mM}$ = 15.4 (Pabst Laboratories, 1956). CPK activity was determined spectrophotometrically in a coupled assay containing 28 mM creatine, 3.3 mM $MgCl_2$, 1 mM ATP, 0.7 mM phosphoenolpyruvate, 0.4 mM NADH, 9 units/mL lactate dehydrogenase, 2.6 units/mL pyruvate kinase, and 82 mM sodium glycine buffer, pH 9.0. Fluorescence measurements were carried out at 30 ± 1 °C in a 1-cm quartz fluorescence cuvette on a Hitachi Perkin-Elmer MPF-2-A spectrofluorometer equipped with a 150-W xenon lamp.

The excitation bandwidth was set at 2 nm, and the emission bandwidth was fixed between 7 and 14 nm. The samples were excited at 300 nm unless stated otherwise. This wavelength is used in order to minimize the inner filter effects by acrylamide, AMP, ADP, and ATP and to ensure that only tryptophan residues in the protein are excited. The fluorescence of a reference solution containing the same concentration of CPK as the sample in 0.24 M NaCl was measured just before measuring the fluorescence of each solution in order to correct for fluctuations in the system. A correction for the inner filter effect of acrylamide was applied to samples containing more than 0.1 M acrylamide. The correction factors for λ_{ex} = 290 nm were 1.044 and 1.24 at 0.05 and 0.24 M acrylamide, respectively (Chignell, 1972).

Quenching measurements were made in 0.05 M Tris–0.03 M acetic acid buffer, pH 8, containing 2–6 nmol mL^{-1} CPK or 60 nmol mL^{-1} L-tryptophan and varying concentrations of the quenching agents KI and acrylamide. In the experiments with KI, quencher concentrations were varied from 0 to 0.24 M. To keep the ionic strength constant at 0.24 M, complemental amounts of NaCl were added to each sample (Lehrer, 1971). In the experiments with acrylamide, the effects of ionic strength were negligible. A small amount of sodium thiosulfate (ca. 100 nmol mL^{-1}) was added to the stock solution of KI to prevent formation of I_3^-.

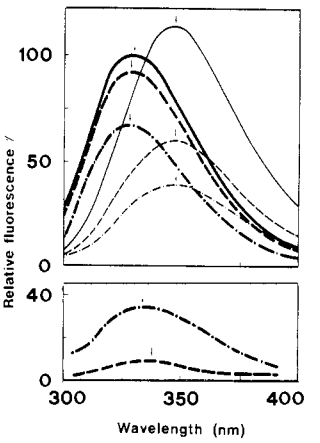

FIGURE 1: Effect of quenching agents on native and denatured CPK. (Top) Native CPK (—), native CPK plus 0.24 M iodide (– –), native CPK plus 0.24 M acrylamide (–·–), CPK in 6 M Gdn·HCl (—), CPK in 6 M Gdn·HCl plus 0.24 M iodide (– –), and CPK in 6 M Gdn·HCl plus 0.24 M acrylamide (–·–). All solutions contained 0.23 mg mL^{-1} CPK and 0.05 M Tris–0.03 M acetic acid buffer, pH 8. Wavelength of excitation was 290 nm. Fluorescence intensity is expressed in percent of the emission at λ_{max} = 330 nm of native CPK. The fluorescence spectra recorded in the presence of acrylamide were corrected for inner filter effect (see Methods). The arrows indicate fluorescence maxima. (Bottom) Difference fluorescence spectra of native CPK vs. CPK quenched by 0.24 M acrylamide (–·–) and of native CPK vs CPK quenched by 0.24 M iodide (– –) (data taken from Figure 1, top). The arrows indicate difference fluorescence peaks.

Results

Figure 1 (top) shows fluorescence emission spectra of native and denatured CPK in the presence and absence of quenching concentrations of iodide and acrylamide. Native CPK exhibits a fluorescence maximum at 330 nm. In the presence of 0.24 M iodide, the maximum is shifted to 328 nm and its amplitude reduced by 7%. Similarly, in the presence of 0.24 M acrylamide there is a shift to 328 nm and a reduction in amplitude by 32%. The spectra of the fluorescence quenched by iodide and acrylamide (Figure 1, bottom) exhibit maxima at 337 and 333 nm, respectively. At these concentrations, iodide and acrylamide reduce CPK activity to 20 and 40% of the control, respectively. However, full activity is regained upon dilution, indicating reversibility of inhibition and, hence, the absence of irreversible structural changes due to the quenching agents. Denaturation of CPK in the presence of 6 M Gdn·HCl shifts the emission maximum to 347 nm and increases the intensity of the band to 113% of the native control. It also increases the susceptibility of the tryptophan residues to quenching. At concentrations of 0.24 M iodide or acrylamide, the fluorescence emission maximum remains unchanged, but its amplitude is reduced to 47 and 35% of the unquenched denatured control, respectively.

Figure 2 shows the effect of increasing concentrations of iodide and acrylamide on tryptophan fluorescence of native and denatured CPK. In this diagram, the ratio of the fluorescence intensity F_0, measured in the absence of the quencher, to the fluorescence intensity F, measured in its presence, is plotted vs. the concentration [q] of the quenching agents (Stern–Volmer plot). With denatured CPK, F_0/F increases linearly with increasing concentrations of iodide, [q], in accordance with the classical Stern–Volmer relationship:

$$F_0/F = 1 + K_{sv}[q] \quad (1)$$

where the slope K_{sv} = 2.8 L mol^{-1} is the Stern–Volmer con-

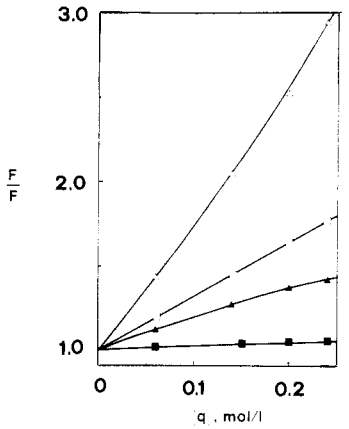

FIGURE 2: Stern–Volmer plots for the quenching of the fluorescence emission of native and denatured CPK by quenching agents: CPK quenched by acrylamide in the presence of 6 M Gdn·HCl (△); CPK quenched by iodide in the presence of 6 M Gdn·HCl (□); CPK quenched by acrylamide (▲); CPK quenched by iodide (■). All solutions contained 0.05 M Tris–0.03 M acetic acid buffer, pH 8. In all experiments the total salt concentration was kept constant at 0.24 M by the addition of appropriate quantities of sodium chloride. The wavelengths of excitation were 290 nm for quenching by iodide and 295 nm for quenching by acrylamide.

stant. Reflecting its greater quenching effectiveness, acrylamide yields a steeper curve with an initial slope of 7.0 and a slight upward curvature. Analogous plots (not shown) are obtained on quenching of L-tryptophan by the two agents, yielding initial slopes of 9.9 and 18.1 with iodide and acrylamide, respectively. The Stern–Volmer plots obtained on quenching of native CPK with the two reagents are much less steep and display a slight downward curvature, suggesting more limited access of the quencher to the tryptophan residues in the folded protein. The nonlinear function describing the course of such Stern–Volmer plots can be written as a modification of the Stern–Volmer law, i.e.

$$F_0/F = 1 + K^P_{sv,q}(\text{eff})[q] \quad (2)$$

where $K^P_{sv}(\text{eff})$ is also a function of the concentration of [q] and is designated the effective Stern–Volmer quenching constant of the protein (Eftink & Ghiron, 1981). When the total tryptophan fluorescence of the protein is considered to be partitioned into a fraction f_a quenchable with a Stern–Volmer constant K_a and a fraction f_b quenchable with a Stern–Volmer constant K_b, the effective quenching constant is

$$K^P_{sv,q}(\text{eff}) = \frac{f_aK_a + f_bK_b + K_aK_b[q]}{1 + (f_aK_b + f_bK_a)[q]} \quad (3)$$

For the limiting case that f_b is completely inaccessible to the quenching agent ($K_b = 0$), this expression is simplified to

$$K^P_{sv,q}(\text{eff}) = \frac{f_aK_a}{1 + f_bK_a[q]} \quad (4)$$

By substituting eq 4 into eq 2 and rearranging the terms, one obtains the modified Stern–Volmer relationship of Lehrer (1971):

$$\frac{F_0}{F_0 - F} = \frac{1}{[q]f_aK_a} + \frac{1}{f_a} \quad (5)$$

Figure 3 demonstrates that plots of the experimental values of $F_0/(F_0 - F)$ vs. $1/[q]$ yield straight lines in accordance with eq 5. Hence, graphical analysis allows evaluation on the parameters f_a and K_a. For quenching of denatured CPK by iodide and acrylamide, the lines intersect with the ordinate at $1/f_a = 1.01$ and 0.99, respectively, indicating the presence of a single class of tryptophan residues of uniform accessibility

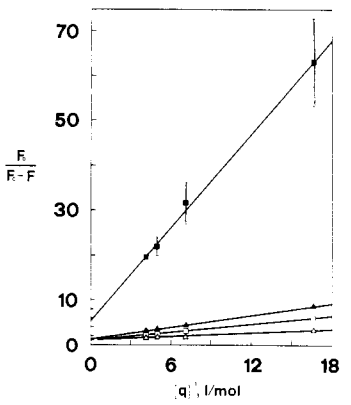

FIGURE 3: Modified Stern–Volmer plots for quenching of the fluorescence emission of native (closed symbols) and denatured (open symbols) CPK by iodide (■, □) and acrylamide (▲, △). Same data as in Figure 2. The symbols refer to the experiments detailed in the caption of Figure 2. The intercepts $1/f_a$ and the slopes $(1/f_a)K_a$ are obtained from the data points by nonlinear regression.

to these quenching agents. In contrast, quenching of native CPK by iodide and acrylamide yields lines intersecting with the ordinate at $1/f_a = 4.2$ and 1.3, respectively. This indicates that of the total tryptophan emission only about 20–25% is affected by iodide while nearly 80% is quenched by acrylamide. The constant, K_a, of the accessible fluorophores determined from the modified Stern–Volmer plot for the quenchable fraction of the fluorescence of native CPK was 0.97 and 2.2 for iodide and acrylamide, respectively. For quenching of denatured CPK by iodide and acrylamide, the corresponding values for K_a are 2.8 and 5.4, respectively, in reasonable agreement with the Stern–Volmer constants derived from the slope of the Stern–Volmer plots of Figure 2 (see above).

The susceptibility of the protein tryptophan fluorescence of CPK to quenching by iodide or acrylamide is markedly influenced by the binding of the adenosine phosphates AMP, ADP, and ATP to the active sites of the enzyme (Figures 4 and 5). Thus, with increasing concentrations of ADP or ATP the effect of iodide on CPK fluorescence is progressively reduced as manifested by the decreasing steepness of the Stern–Volmer plots (Figure 4, middle and bottom). The effect is independent of the excitation wavelength. At saturating concentrations of the two coenzymes, quenching by iodide is completely abolished. A similar effect is observed on binding of AMP to CPK (Figure 4, top). However, in contrast to the complexes with the di- and triphosphate homologues, there is some residual quenchability of tryptophan fluorescence by iodide, even in the fully formed complex. In an entirely analogous fashion, nucleoside phosphate binding also reduces the quenchability of CPK tryptophan fluorescence by acrylamide (Figure 5). However, in all three cases, the quenching effect of acrylamide is reduced only in part.

The effect of the nucleoside phosphates on the Stern–Volmer plots of CPK tryptophan fluorescence quenching by iodide and acrylamide can be utilized to assess binding of these ligands to CPK. As shown in the Appendix at any given concentration of the quenching agent [q], the ratio of the Stern–Volmer increments $(F/F_0 - 1)_{q,L} = Q_{q,L}$ and $(F/F_0 - 1)_{q,L=0} = Q^P_q$ measured in the presence and absence of the ligand L, respectively, can be described by a hyperbolic function of the ligand concentration [L]. Its analytical expression (eq 10) is formally identical with that of a binding isotherm. Thus, a plot of experimental $Q_{q,L}/Q^P_q$ values vs. log [L] yields sigmoidal fluorescence quenchability titration curves (Figure 6). The parameters $[L]_{50,q}$ and n_q, representing the ligand concentration at the midpoint of the titration curve and the limiting fractional

Table I: Evaluation of Dissociation Constants of Complexes of Adenosine Phosphates with CPK from Fluorescence Quenchability Titration Measurements[a]

ligand	concn of quencher employed, [q] (M)[b]	maximum Stern–Volmer increment of CPK at [q], Q_q^P	fractional Stern–Volmer increment at [q] and ligand saturation, n_q[c]	residual fractional fluorescence of CPK ligand complex, R[d]	ligand concn yielding half-maximum quenching, $[L]_{50,q}$ (M)[c]	dissociation constant, K_D (M)[e]
AMP	0.1 iodide	0.039	0.52	0.85	2.0×10^{-3}	1.7×10^{-3}
ADP	0.24 iodide	0.093	0	0.86	1.4×10^{-4}	1.3×10^{-4}
ATP	0.15 iodide	0.057	0	0.90	5.2×10^{-4}	4.9×10^{-4}
AMP	0.11 acrylamide	0.22	0.61	0.85	1.0×10^{-3}	9.5×10^{-4}
ADP	0.11 acrylamide	0.21	0.61	0.86	1.9×10^{-4}	1.7×10^{-4}
ATP	0.11 acrylamide	0.23	0.77	0.90	5.6×10^{-4}	5.2×10^{-4}

[a] The values employed for the calculations were taken from the interpolated quenching curves of Figures 4 and 5. [b] Concentration of quenching agent at which binding parameters were evaluated. [c] Obtained by least-squares fitting to a function of the form of eq 13 in the Appendix. [d] Values determined for static quenching of CPK fluorescence by adenosine phosphates by Mertens (1978). [e] Evaluated from $[L]_{50,q}$ and n_q according to eq 14 of the Appendix. The dissociation constants determined by direct fluorometric titration of complexes of CPK with AMP, ADP, and ATP under comparable conditions were 3.1×10^{-3} M, 1.6×10^{-4} M, and 8.3×10^{-4} M, respectively (Mertens, 1978); the values obtained by gradient sedimentation at pH 7.9, 3 °C, were 1×10^{-4} M for ADP and 5×10^{-4} M for ATP (Kuby et al., 1962).

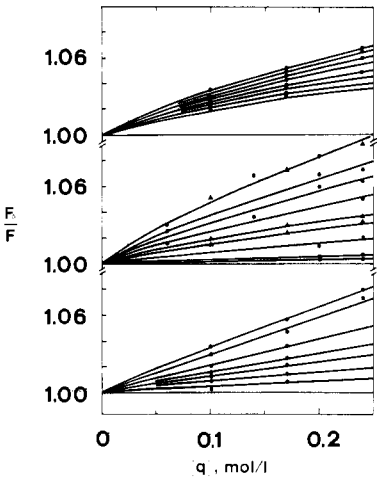

FIGURE 4: Influence of nucleoside phosphate binding on the susceptibility of fluorescence emission of CPK to quenching by iodide. Stern–Volmer plots for quenching by iodide were obtained at various concentrations of nucleoside phosphates. The lines were calculated from data points by least-squares fitting. (Top) Effect of AMP binding. AMP concentrations from top to bottom: none and 1.4×10^{-4}, 3.7×10^{-4}, 5.5×10^{-4}, 9×10^{-4}, 4×10^{-3}, and 7×10^{-3} M. λ_{ex} = 300 nm (●). (Middle) Effect of ADP binding. ADP concentrations from top to bottom: none and 2.8×10^{-5}, 5.6×10^{-5}, 1.12×10^{-4}, 1.5×10^{-4}, 3.5×10^{-4}, 4.5×10^{-4}, 6.7×10^{-4}, and 1.0×10^{-3} M. λ_{ex} = 290 nm (●); λ_{ex} = 300 nm (▲). (Bottom) Effect of ATP binding. ATP concentrations from top to bottom: none and 3.3×10^{-5}, 3.3×10^{-4}, 4.6×10^{-4}, 6.5×10^{-4}, 1.8×10^{-3}, and 2.9×10^{-3} M. λ_{ex} = 300 nm (●). Conditions were as given under Methods; the salt concentration was kept constant at 0.24 M by the addition of appropriate quantities of sodium chloride.

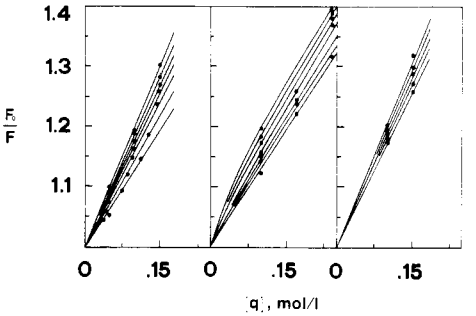

FIGURE 5: Influence of ligand binding on susceptibility of CPK fluorescence emission to quenching by acrylamide. Stern–Volmer plots for quenching by acrylamide were obtained at various concentrations of nucleoside phosphates. The lines were calculated from data points by least-squares fitting. (Left) Effect of AMP binding. AMP concentrations from top to bottom: none and 1.4×10^{-4}, 3.7×10^{-4}, 5.5×10^{-4}, 9×10^{-4}, 3×10^{-3}, and 7×10^{-3} M. (Center) Effect of ADP binding. ADP concentrations from top to bottom: none and 1.9×10^{-5}, 5.8×10^{-5}, 1.5×10^{-4}, 2.4×10^{-4}, 4.2×10^{-4}, and 1×10^{-3} M. (Right) Effect of ATP binding. ATP concentrations from top to bottom: none and 1.2×10^{-4}, 3×10^{-4}, 6.8×10^{-4}, and 1.9×10^{-3} M. Conditions: λ_{ex} = 300 nm; emission data were corrected for inner filter effect of acrylamide (see Methods); the salt concentration was kept constant at 0.24 M sodium chloride.

Stern–Volmer increment at saturation, respectively, are determined by least-squares fitting (eq 14) and are listed in Table I. The values of $[L]_{50,q}$ obtained from the complexes with a given nucleoside phosphate and iodide or acrylamide as the quenching agent are fairly comparable. This is also documented in Figure 7, where the data obtained on titration of CPK with ADP and ATP are replotted according to eq 11, yielding normalized fluorescence quenchability titration curves. From the measured $[L]_{50,q}$ values, the dissociation constants K_D are calculated as indicated in the caption of Table I. They are slightly smaller than the $[L]_{50,q}$ values. For comparison, the dissociation constants determined by gradient sedimentation (Kuby et al., 1962) and by direct fluorescence quenching titration with the nucleoside phosphates (Mertens, 1978) are also included.

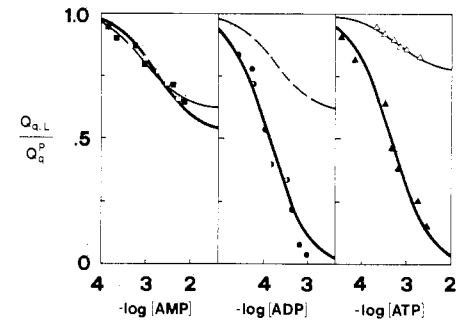

FIGURE 6: Fluorescence quenchability titration of binding of adenosine phosphates to CPK. Fractional Stern–Volmer increments $Q_{q,L}/Q_q^P$, derived from quenching experiments with iodide (Figure 4) and with acrylamide (Figure 5), are plotted as a function of the negative logarithm of the molar adenosine phosphate concentration. (Left) Effect of AMP binding on quenching by 0.1 M iodide (□) and by 0.11 M acrylamide (■); λ_{ex} = 300 nm. (Center) Effect of ADP binding on quenching by 0.24 M iodide using λ_{ex} = 290 nm (●) and λ_{ex} = 300 nm (◑) and by 0.11 M acrylamide using λ_{ex} = 300 nm (○). (Right) Effect of ATP binding on quenching by 0.15 M iodide (▲) and by 0.11 M acrylamide (△); λ_{ex} = 300 nm. The titration curves for quenching by iodide (fat line) and by acrylamide (thin line) were obtained by least-squares fitting of the experimental data to eq 13 of the Appendix.

DISCUSSION

CPK displays a typical tryptophan fluorescence emission spectrum. The emission maximum at 330 nm in native CPK and its bathochromic shift to 347 nm upon denaturation have

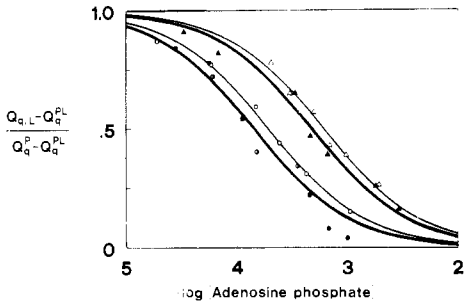

FIGURE 7: Normalized fluorescence quenchability titration curves for ADP and ATP binding to CPK. The normalized Stern–Volmer increments, $(Q_{q,L} - Q_q^{PL})/(Q_q^P - Q_q^{PL})$, derived from quenching experiments with iodide (Figure 4) and acrylamide (Figure 5) were determined according to eq 11 of the Appendix and plotted as a function of the negative logarithm of the molar ADP and ATP concentration. Binding of ADP: iodide quenching, λ_{ex} = 290 nm (●), λ_{ex} = 300 nm (◑); acrylamide quenching, λ_{ex} = 300 nm (○). Binding of ATP: iodide quenching, λ_{ex} = 300 nm (▲); acrylamide quenching, λ_{ex} = 300 nm (△). The titration curves for quenching by iodide (fat line) and by acrylamide (thin line) were determined by least-squares fitting of the data points to eq 11 of the Appendix.

also been observed by Yao et al. (1982). It indicates that in native CPK most of the tryptophan residues are located in the interior of the molecule (Eftink & Ghiron, 1976). This is also supported by the very low susceptibility of the fluorescence to quenching by iodide (Lehrer, 1971), suggesting that only a fraction of the four tryptophan residues is accessible to the aqueous phase. This partitioning is likewise reflected by the bathochromic shift of the maximum of the difference fluorescence spectrum (λ_{max} = 337 nm) relative to that of the residual fluorescence spectrum (λ_{max} = 328 nm) (Figure 1). According to Burstein (1973), the spectral position of the emission maximum of protein tryptophan residues is determined by their dielectric environment and can be grouped into three types (Vedenkina et al., 1972). Indole side chains completely buried within the apolar protein matrix display their fluorescence maximum between 320 and 330 nm (type I), those with partial access to the polar solvent are centered at about 340 nm (type II), and those that are completely exposed are at 350 nm (type III). The location at the difference emission band at 337 nm suggests that the tryptophan residue quenched by iodide belongs to type II. An identically located difference emission band was observed earlier in CPK quenched by complex formation with the coenzyme ADP (Mertens, 1978; Vašák et al., 1979).

Although acrylamide is an intrinsically more powerful quenching agent for tryptophan than is iodide, this agent is less suitable to differentiate tryptophan residues topographically since this uncharged molecule, in contrast to iodide, is able to penetrate the protein and to quench interior residues as well (Eftink & Ghiron, 1976; Lehrer, 1971; Lehrer & Leavis, 1978). Thus, while its effectiveness to quench the overall tryptophan fluorescence in native CPK is 4–5 times larger than that of iodide, there is only a 5-nm separation between the maximum of the difference fluorescence spectrum (333 nm) and of the residual fluorescence spectrum (328 nm) (Figure 1).

In several proteins it has been demonstrated that fluorescence quenching by iodide and acrylamide is of the collisional type (Eftink & Ghiron, 1975; Lehrer, 1971). While no explicit proof can be given here, it seems likely that such a mechanism pertains also to the present system. This is supported in particular by the close fit of the fluorescence quenching data of denatured CPK by iodide with the Stern–Volmer law (Stern & Volmer, 1919) as shown in Figure 2. Similar linear plots are obtained for iodide quenching of tryptophan. The slight upward curvature on quenching of denatured CPK by acrylamide is also seen with tryptophan and is believed to reflect a small contribution of static quenching to the dynamic quenching of the excited indole chromophore by this agent (Eftink & Ghiron, 1976; Keizer, 1983; Lehrer & Leavis, 1978).

The downward curvatures observed in the Stern–Volmer plots of the native enzyme quenched by iodide and acrylamide are a consequence of the unequal accessibility of the fluorophores to the quenching agent and are commonly seen in proteins containing more than one class of tryptophans (Eftink & Ghiron, 1976; Einarson, 1977).

The presentation of the quenching data according to Lehrer's modified Stern–Volmer equation (Figure 3) confirms that quenching by iodide in contrast to quenching by acrylamide affects only a small fraction of the total tryptophan fluorescence of native CPK (Lehrer, 1971). Although in the absence of detailed information on the quantum yield of the different tryptophan residues the fractional fluorescence provides no measure of the number of fluorophores exposed to iodide (Eftink & Ghiron, 1976), it is tempting to suggest that in native CPK iodide quenches only one of the four tryptophan residues (f_a = 0.20–0.25) while acrylamide affects the fluorescence of more residues (f_a = 0.8). That under denaturing conditions both iodide and acrylamide gain access to all tryptophan residues is borne out by the fact that Lehrer's modified Stern–Volmer plots intersect at $F_0/(F_0 - F) = 1$, i.e., $f_a = 1$.

That in native CPK only a single partially exposed tryptophan residue is affected by iodide is also supported by the complete abolition of its quenching effect by the binding of ADP or ATP (Figure 4). This loss of susceptibility to quenching can be accounted for by several not mutually exclusive mechanisms. Thus, a partial explanation is provided by the fact that fluorescence of the same tryptophan residue is already quenched by the binding of the nucleoside phosphates to the enzyme. Mertens (1978) has shown that the protein fluorescence of native CPK is reduced in the presence of saturating concentrations of adenosine phosphates by 9–15%, and Vašák et al. (1979) suggested the possibility that this effect is caused by resonance transfer from a tryptophan to the adjacent adenine chromophore of the ligand. Another explanation for the effect of the two coenzymes on iodide quenching is that the bulky nucleoside phosphate reduces the exposure of the susceptible tryptophan residue to the quenching agent either by direct shielding or by a conformational change induced by the ligand. An example of such a shielding effect has been reported for apolipoprotein where dynamic fluorescence quenching is markedly reduced by phospholipid binding (Pownall & Smith, 1974).

A somewhat analogous interference of adenosine phosphate binding with quenching by iodide has been reported for the coenzyme fluorescence of glycogen phosphorylase (Honikel & Madsen, 1973). Quenching differs for the phosphorylases *a* and *b* and is affected differently by ligand binding to the two forms. While the allosteric inhibitors ATP and glucose 6-phosphate render the fluorescence of the pyridoxal phosphate moiety of phosphorylase *b* more accessible, the activator, AMP, and the substrate, glucose 1-phosphate, together cause it to be totally inaccessible to fluorescence quenching by iodide. AMP and glucose 1-phosphate are believed to mediate a conformational change that "buries" the coenzyme.

The concentration dependence of the ability of the nucleoside phosphates to suppress the susceptibility of tryptophan

residues in CPK toward quenching agents provides a means to assess the binding equilibria of the enzyme with these ligands. Although the extent of fluorescence quenching by iodide and acrylamide in this system is small (Figures 4 and 5), titration with these ligands produces changes in the Stern–Volmer plots that are in accordance with the extent of binding site occupation. The resulting titration curves (Figures 6 and 7) display classical saturation behavior and yield association constants (Table I) that are in fair agreement with values obtained from other measurements (Mertens, 1978; Kuby et al., 1962).

Appendix

In the presence of a ligand L that binds to a protein with a dissociation constant K_D and that on binding reduces protein fluorescence by static quenching to a residual fractional fluorescence R, the resulting total fluorescence F_0 of the sample is

$$F_0 = \frac{K_D}{K_D + [L]}F_{00} + \frac{[L]}{K_D + [L]}RF_{00} \tag{6}$$

where F_{00} is the total fluorescence in the absence of the ligand and [L] is the concentration of the ligand. On addition of the quenching agent q to the system, the total fluorescence intensity F is further reduced to

$$F = \frac{K_D}{K_D + [L]}\frac{F_{00}}{1 + K^P_{sv,q}(\text{eff})[q]} + \frac{[L]}{K_D + [L]}\frac{RF_{00}}{1 + K^{PL}_{sv,q}(\text{eff})[q]} \tag{7}$$

where $K^P_{sv,q}(\text{eff})$ and $K^{PL}_{sv,q}(\text{eff})$ are the effective Stern–Volmer constants for quenching of the fluorescence of the free protein (P) and of the protein–ligand complex (PL), respectively, as defined in eq 2. The ratio of the total fluorescence measured in the absence (eq 6) and in the presence (eq 7) of the quencher is

$$\frac{F_0}{F} = \frac{K_D + [L]R}{K_D\dfrac{1}{1 + K^P_{sv,q}(\text{eff})[q]} + [L]R\dfrac{1}{1 + K^{PL}_{sv,q}(\text{eff})[q]}} \tag{8a}$$

Rearranging this expression to a Stern–Volmer type formula yields

$$\frac{F_0}{F} = 1 + \left[K_D\left(1 - \frac{1}{1 + K^P_{sv,q}(\text{eff})[q]}\right) + [L]R\left(1 - \frac{1}{1 + K^{PL}_{sv,q}(\text{eff})[q]}\right)\right] \Bigg/ \left[K_D\frac{1}{1 + K^P_{sv,q}(\text{eff})[q]} + [L]R\frac{1}{1 + K^{PL}_{sv,q}(\text{eff})[q]}\right] \tag{8b}$$

By introducing into eq 8a the substitutions $K^P_{sv,q}(\text{eff})[q] = Q^P_q$ and $K^{PL}_{sv,q}(\text{eff})[q] = Q^{PL}_q = n_qQ^P_q$, where at a given concentration of q the values of Q^P_q and Q^{PL}_q are constants and n_q is their ratio (see below), one obtains for the actual Stern–Volmer increment $Q_{q,L} = (F_0/F) - 1$ in function of [L] the expression

$$Q_{q,L} = Q^P_q\frac{K_D + n_q[L]R\dfrac{1 + Q^P_q}{1 + n_qQ^P_q}}{K_D + [L]R\dfrac{1 + Q^P_q}{1 + n_qQ^P_q}} \tag{9}$$

In the absence of ligand the increment assumes the maximum value Q^P_q and at $[L] \gg K_D$ the minimum value $Q^{PL}_q = n_qQ^P_q$. Rearrangement of eq 9 yields the fractional Stern–Volmer increment:

$$\frac{Q_{q,L}}{Q^P_q} = \frac{K_D + n_q[L]R\dfrac{1 + Q^P_q}{1 + n_qQ^P_q}}{K_D + [L]R\dfrac{1 + Q^P_q}{1 + n_qQ^P_q}} \tag{10a}$$

$$\frac{Q_{q,L}}{Q^P_q} = \frac{K_D}{K_D + [L]R\dfrac{1 + Q^P_q}{1 + n_qQ^P_q}}(1 - n_q) + n_q \tag{10b}$$

Its values range from $Q_{q,L}/Q^P_q = 1$ in the absence of ligand to $Q_{q,L}/Q^P_q = n_q$ at saturating concentrations of ligand. By a further rearrangement of eq 10a/10b, one obtains the normalized fractional Stern–Volmer increment:

$$\frac{(Q_{q,L}/Q^P_q) - n_q}{1 - n_q} = \frac{Q_{q,L} - Q^{PL}_q}{Q^P_q - Q^{PL}_q} = \frac{K_D}{K_D + [L]R\dfrac{1 + Q^P_q}{1 + n_qQ^P_q}} \tag{11}$$

Equation 11 resembles that of the binding isotherm for the reaction

$$P + L \rightleftharpoons PL$$

$$\frac{[P]}{[P] + [PL]} = \frac{[P]}{[P_{tot}]} = \frac{K_D}{K_D + [L]} \tag{12}$$

where $[P_{tot}]$ is the total protein concentration. The two expressions are identical under conditions where $R = 1$ and $n_q = 0$ and where Q^P_q is very small. When the two functions are plotted vs. log [L], they yield identical sigmoidal titration curves that are shifted, however, with respect to each other along the log [L] axis by the magnitude $|\log [R(1 + Q^P_q)/(1 + n_qQ^P_q)]|$. Practically, the parameters of a fluorescence quenchability titration curve are determined by fitting a function of the form

$$\frac{Q_{q,L}}{Q^P_q} = \frac{[L]_{50,q} + n_q[L]}{[L]_{50,q} + [L]} \tag{13}$$

to the experimental values $Q_{q,L_i}/Q^P_q$ measured at fixed concentration of the ligand $[L_i]$. Equation 13 is equivalent to eq 10. $[L]_{50,q}$ is the ligand concentration exerting half-maximum reduction in quenchability, i.e., $(Q_{q,L} - Q^{PL}_q)/(Q^P_q - Q^{PL}_q) = 0.5$. $[L]_{50,q}$ and n_q are the parameters pertaining to measurements at a fixed concentration of q. They are evaluated by the least-squares procedure, i.e., by searching for the minimum of the expression

$$\sum_{i=1}^{m}\left(\frac{Q_{q,L_i}}{Q^P_q} - \frac{[L]_{50,q} + n_q[L_i]}{[L]_{50,q} + [L_i]}\right)^2$$

The dissociation constant K_D of the protein ligand complex is given by the relationship

$$K_D = [L]_{50,q}R\frac{1 + Q^P_q}{1 + n_qQ^P_q} \tag{14}$$

Registry No. CPK, 9001-15-4; ATP, 56-65-5; ADP, 58-64-0; AMP, 61-19-8; iodide, 20461-54-5; acrylamide, 79-06-1; tryptophan, 73-22-3.

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Coenzyme A Biosynthesis: Steric Course of 4′-Phosphopantothenoyl-L-cysteine Decarboxylase†

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ABSTRACT: 4′-Phosphopantothenoyl-L-cysteine decarboxylase (*P*PC decarboxylase) was partially purified from rat liver. 4′-Phosphopantothenoyl[2-$^2H_1$]-L-cysteine was synthesized and converted by *P*PC decarboxylase to 4′-phospho[1-$^2H_1$]pantetheine. The product was degraded by reduction with Raney nickel followed by acidic hydrolysis to [1-$^2H_1$]ethylamine. The latter was converted to the (−)-camphanamide derivative, NMR studies of which revealed that the deuterium was located in the *pro*-1*S* position. Also, unlabeled 4′-phosphopantothenoyl-L-cysteine was incubated with *P*PC decarboxylase in D_2O, giving, after degradation, the (−)-camphanamide of (1*R*)-[1-$^2H_1$]ethylamine. The results show that the decarboxylation takes place with retention of configuration. These results are discussed in terms of possible mechanisms for the decarboxylation.

The decarboxylation of 4′-phosphopantothenoyl-L-cysteine (*P*PC)[1] (**1**) (see Chart I) to 4′-phosphopantetheine (*P*P) (**2**) by 4′-phosphopantothenoyl-L-cysteine decarboxylase [4′-phospho-*N*-(D-pantothenoyl)-L-cysteine carboxy-lyase, EC 4.1.1.36] (*P*PC decarboxylase) constitutes a step in the biosynthesis of the ubiquitous acyl group carrier coenzyme A (Abiko, 1975). Two purifications of this enzyme have been reported, one using rat liver (Abiko, 1970, 1967) and the other horse liver (Scandurra et al., 1974) as source. The decarboxylases obtained from the two sources appeared to be very similar. The enzyme has an absolute requirement for the 4′-phosphate of the substrate and does not decarboxylate pantothenoylcysteine. We now report the results of our studies on the steric course of the decarboxylation of *P*PC, which show that the decarboxylation proceeds in a *retention* mode.

Materials and Methods

^1H NMR spectra were run on a Varian EM-360 and on a Bruker WM-250 instrument. ^2H NMR spectra were run on

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[1] Abbreviations: *P*PC decarboxylase, 4′-phosphopantothenoyl-L-cysteine decarboxylase; *P*PC, 4′-phosphopantothenoyl-L-cysteine; *P*P, 4′-phosphopantetheine; Tris, tris(hydroxymethyl)aminomethane; SW, spectral width; PW, pulse width; RD, relaxation delay; LB, line broadening.

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