Mechanistic Studies with General Acyl-CoA Dehydrogenase and Butyryl-CoA Dehydrogenase: Evidence for the Transfer of the β-Hydrogen to the Flavin N(5)-Position as a Hydride†

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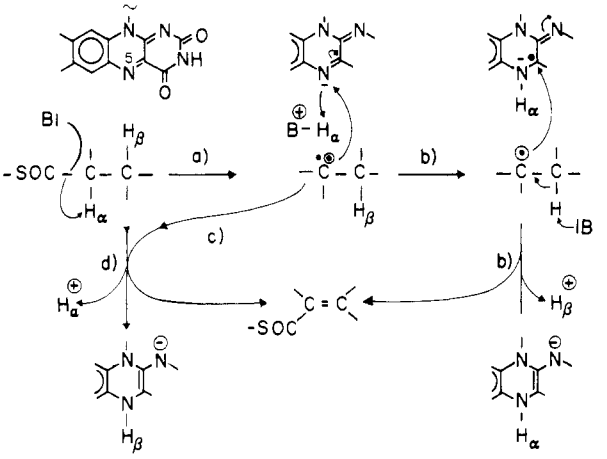
ABSTRACT: Butyryl-CoA dehydrogenase from *Megasphera elsdenii* catalyzes the exchange of the α- and β-hydrogens of substrate with solvent [Gomes, B., Fendrich, G., & Abeles, R. H. (1981) *Biochemistry 20*, 1481–1490]. The stoichiometry of this exchange was determined by using ^3H_2O label as 1.94 ± 0.1 per substrate molecule. The rate of ^3H label incorporation into substrate under anaerobic conditions is monophasic, indicating that both the α- and β-hydrogens exchange at the same rate. The exchange in ^2H_2O leads to incorporation of one ^2H each into the α- and the β-positions of butyryl-CoA, as determined by companion ^1H NMR experiments and confirmed by mass spectroscopic analysis. In contrast, with general acyl-CoA dehydrogenase from pig kidney, only exchange of the α-hydrogen was found. The β-hydrogen is the one that is transferred (reversibly) to the flavin 5-position during substrate dehydrogenation. This was demonstrated by reacting 5-^3H- and 5-^2H-reduced 5-deaza-FAD–general acyl-CoA dehydrogenase with crotonyl-CoA. Only one face of the reduced flavin analogue is capable of transferring hydrogen to substrate. The rate of this reaction is 11.1 s^{-1} for 5-deaza-FAD–enzyme and 2.2 s^{-1} for [5-^2H]deaza-FAD–enzyme, yielding an isotope effect of 5. These values compare with a rate of 2.6 s^{-1} for the reaction of native reduced enzyme with crotonyl-CoA. The two reduced enzymes (normal vs. 5-deaza-FAD–enzyme) thus react at similar rates, indicating a similar mechanism. The results are interpreted as evidence for a catalytic sequence in which the α-hydrogen is abstracted as a proton, followed by expulsion of the β-hydrogen as a hydride and its direct transfer to the flavin position N(5).

Flavin-dependent acyl-CoA dehydrogenases, such as butyryl-CoA dehydrogenase and general acyl-CoA dehydrogenase, catalyze the α–β-dehydrogenation of fatty acid thio esters to the corresponding *trans*-enoyl-CoA analogue with removal of *pro-2R* and *pro-3R* hydrogens (Table I; Biellman & Hirth, 1970a,b; Buckler et al., 1970). This reaction is probably initiated by abstraction of the relatively acidic α-hydrogen as a proton to form a transient carbanionic species (Scheme I, step a). Evidence supporting this concept comes from experiments with modified substrates. Thus, for example, vinylacetyl-CoA undergoes a 2 → 4 tautomerization, and halide is eliminated from 3-fluoro substrates in the presence of butyryl-CoA dehydrogenase (Fendrich & Abeles, 1982). Acyl-CoA dehydrogenases are irreversibly inactivated by 3-ynoyl thio ester derivatives, a process proposed to occur via isomerization to the 2,3-allenyl derivatives (Frerman et al., 1980; Fendrich & Abeles, 1982). The allenic substrate analogue 3,4-pentadienoyl-CoA is isomerized in the presence of general acyl-CoA dehydrogenase to the 2,4-diene, a reaction that could reasonably occur via abstraction of the α-hydrogen as a proton (Wenz et al., 1982). This same reaction appears to involve nucleophilic addition of the mesomeric intermediate carbanion to from an unstable N(5)-reduced flavin adduct (Wenz et al., 1982). Finally, experiments with long-chain acyl-CoA substrate analogues, in which the thio ester group is replaced by either a thioether or a ketone functional group, also provide data consistent with a carbanion mechanism (Thorpe et al., 1981). The question about the second half of the reductive half-reaction, i.e., the rupture of the substrate

Scheme I



β-C–H bond and the transfer of the redox equivalents to the flavin, has, to our knowledge, first been addressed experimentally in the present work and in a parallel model study by Farng & Bruice (1983). An earlier proposal by Cornforth (1959) was that the initial carbanion is oxidized via radical steps. (Scheme I, step b). An alternative possibility is that reduction of the flavin represents addition of a hydride to the N(5)-position (Scheme I, step c). Such a reaction might be concerted with the initial abstraction of the α-proton (Scheme I, step d). Third, a covalent adduct could be formed by addition of the carbanion to the flavin or by collapse of the flavin–substrate radical pair, followed by fragmentation of the adduct to yield the final products. For a detailed discussion of these mechanistic alternatives, refer to pertinent review articles (Bright & Porter, 1975; Hemmerich, 1976; Walsh, 1980; Bruice, 1980; Ghisla, 1982). One aspect of the dehydrogenation reaction that is addressed in the present paper is whether the α- or β-hydrogens of the bound substrate are transferred (directly) to the flavin. Depending on the type of

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Table I: Exchange of α- and β-Hydrogens Catalyzed by Acyl-CoA Dehydrogenases and Enoate Reductase

enzyme	source	exchange	relative rate of exchange	ref
butyryl-CoA dehydrogenase	pig liver	α and β	$\alpha \gg \beta$	Biellmann & Hirth (1970a,b)
butyryl-CoA dehydrogenase	pig liver	nd[a]	nd	Bucklers et al. (1970)
butyryl-CoA dehydrogenase	*Clostridium kluyveri*	none		Bühler (1981)
enoate reductase	*Clostridium* La1	none		Bühler (1981)
butyryl-CoA dehydrogenase	*Megasphaera elsdenii*	α and β	nd	Gomes et al. (1981)
butyryl-CoA dehydrogenase	*Megasphaera elsdenii*	α and β	$\alpha = \beta$	this work
general acyl-CoA dehydrogenase	ox liver	α and β	$\alpha \gg \beta$	Murfin (1974)
general acyl-CoA dehydrogenase	rat liver	α		Ikeda et al. (1983)
general acyl-CoA dehydrogenase	pig kidney	α		this work

[a] nd = not determined.

mechanism, either the β-hydrogen (Scheme I, steps d or a + c) or the α-hydrogen (Scheme I, steps a + b) might be transferred. If the carbanionic species has a finite lifetime (and if the proton-abstracting enzyme base is exposed to solvent), then exchange of the α-hydrogen with solvent might occur. Preferential exchange of a single hydrogen might result also at the level of the reduced enzyme–product complex if the proton-abstracting base and the reduced flavin have different degrees of exposure to the solvent. Finally, exchange of both hydrogens might occur at the level of free reduced enzyme and product by a mechanism which will be detailed below.

The exchange of the α- *and* β-hydrogens in the presence of acyl-CoA dehydrogenases has been reported by various authors (cf. also Table I below). Biellmann & Hirth (1970a,b) and Murfin (1974) suggested that the α-hydrogen exchanges faster than the β-hydrogen, without, however, providing conclusive kinetic evidence. A determination of the relative rate of exchange of the two hydrogens, together with complementary kinetic data on relevant catalytic steps, might thus yield answers to some of the questions outlined above.

Materials and Methods

Materials. General acyl-CoA dehydrogenase was isolated from pig kidneys as described earlier (Thorpe et al., 1979; Gorelick, 1982). Butyryl-CoA dehydrogenase was isolated from *Megasphaera elsdenii* (Engel, 1981). Protocatechuate dioxygenase was used as the oxygen scavenger and was a gift from Dr. D. P. Ballou, The University of Michigan. Glucose oxidase was from *Aspergillus niger* (Swoboda & Massey, 1965). *n*-Butyryl-CoA, lithium salt (lot 958201), and crotonyl-CoA, lithuim salt (lot 115836), were from P-L Biochemicals, Milwaukee, WI. Tritiated water was from Amersham. NaB^3H_4 (lot 1576-211) was from NEN. NaB^2H_4 (98% ^2H) was from Sigma. Aquasolve scintillation fluid was from Amersham or NEN.

Instrumentation. Optical spectra were recorded with Cary 17 or 219 recording spectrophotometers. Radioactivity was measured with a Packard Tricarb instrument; selected vials were calibrated internally; the average counting efficiency was ~30%. The presence of HCl, NaOH, or protein was not found to affect the counting efficiency in the system used. Rapid-reaction studies were carried out with a stopped-flow spectrophotometer interfaced with a Nova 2 (Data General) minicomputer system, which was designed and built by Dr. D. P. Ballou, The University of Michigan. This instrument is described in more detail by Beaty & Ballou (1981). NMR measurements were carried out with a Bruker FT 360 instrument, with benzene-d_6 as an internal lock, or with a Bruker AM 250 instrument, using ^2H_2O as an internal lock. Mass spectroscopic analysis was carried out on a Finnigan 3200 mass spectrometer linked with a Finnigan INCOS 2000 data system, either by direct insertion or after gas chromatographic analysis on a Finnigan 9500 instrument connected on line with the mass spectrophotometer.

Incorporation of ^3H into Butyryl-CoA. In a typical experiment, an incubation mixture contained 800 nmol of butyryl-CoA, 0.2 nmol (1 IU) of butyryl-CoA dehydrogenase, 2.5 μmol of protocatechuate, and 2 milliunits of protocatechuate dioxygenase in 0.1 mL of 0.05 M phosphate buffer, pH 7.6, containing 4.6×10^8 cpm ^3H_2O (83 cpm/nmol of water). The incubation mixture was first deoxygenated with a gentle nitrogen stream blown over the surface for 5 min. After the addition of butyryl-CoA dehydrogenase, the container was kept closed to prevent access of oxygen. At given time intervals (cf. Figure 1), 10-μL aliquots were removed and quenched immediately by addition to 10 μL of 10% sodium dodecyl sulfate to denature the protein. Each denatured sample was applied to a small column (0.5-mL bed volume) of Dowex anionic exchanger (OH^- form). Columns were washed subsequently with four 1.0-mL aliquots of distilled water (which eluted >99% of the non chemically bound ^3H) and then with a further 20–50 mL of water until the eluted counts had reached background level. Subsequent washing with three or four 1-mL aliquots of 1 M HCl eluted any radioactivity bound in anionic form (e.g., CoA derivatives). The fractions containing radioactivity were counted as described below. This experimental protocol was employed for the kinetic incorporation measurements. For determination of the stoichiometry of tritium incorporation, 2 nmol of butyryl-CoA dehydrogenase was employed, and the reaction was stopped after 10–20 min by addition of sodium dodecyl sulfate to 10% final concentration. Control experiments were performed with the same reaction mixture minus butyryl-CoA dehydrogenase.

Preparation of [2,3-^3H]Butyryl-CoA. A similar procedure to that described above was employed, using, however, the glucose/glucose oxidase/catalase system for scavenging oxygen (Swoboda & Massey, 1965), 1 μmol of butyryl-CoA, and ^3H_2O at a specific activity of 1700 cpm/nmol. Products were separated by using a 1.0-mL Dowex anionic exchanger (OH^-) employing the same elution method. Fractions with a constant ratio of radioactivity/A_{260} were combined and used for the tritium-release experiments described below.

Preparation of [2(R),3(R)-$^2H_2$]Butyryl-CoA. Butyryl-CoA dehydrogenase from *M. elsdenii* (20 nmol) was made up in 1 mL of 0.05 M phosphate buffer, prepared from P_2O_5 dissolved in 99.7% ^2H_2O and adjusted to "pH" 7.5 with anhydrous K_2CO_3. The enzyme was incubated anaerobically at 25 °C with 18 mg (~19 μmol) of butyryl-CoA for 12 h. The reaction mixture was then heated in a boiling water bath for 4 min while still anaerobic. Denaturated protein was removed by filtration through an Amicon PM 10 membrane, and the filtrate was lyophilized.

For the NMR analysis, ~5 μmol of the product was in-

Table II: Release of α- and β-Hydrogens from Butyryl-CoA and Butyric Acid

conditions	time (h)	$CH_3CH^3HCH_2COOR^a$ (cpm)		$CH_3CH^3HCH^3HCOOR^b$ (cpm)	
		H_2O fraction	HCl fraction	H_2O fraction	HCl fraction
incubation with butyryl-CoA dehydrogenase (O_2 as acceptor)	0.5	3530 (98.5)[c]	50 (1.5)		
control (no enzyme)	0.5	40 (2.5)	1520 (97.5)		
12 N KOH, 110 °C	0	30 (3)	1010 (97)	30 (1.5)	1970 (98.5)
12 N KOH, 110 °C	15	130 (9)	1320 (91)	500 (37)	850 (63)
12 N KOH, 110 °C	40	80 (7)	1130 (93)	760 (40)	1140 (60)

[a] Obtained from the reaction of crotonyl-CoA with reduced 5-deaza-FAD–general acyl-CoA dehydrogenase, in which the 5-deaza-FAD was reduced with NaB^3H_4 and then incorporated into apoenzyme (see text for details). The fractions were obtained as described under Materials and Methods. [b] Obtained by exchange of solvent ^3H_2O with butyryl-CoA dehydrogenase from *M. elsdenii* (see text for details). [c] Values in parentheses are percentages.

cubated at 25 °C for 12 h with 0.1 N KOH. The solution was then adjusted to pH 1 with phosphoric acid and extracted 3 times with 0.5-mL aliquots of $^2HCCl_3$. The chloroform extracts were dried over $MgSO_4$, and the solvent was exchanged with CCl_4 by repeated evaporation.

Preparation of Reduced [5-^3H]Deaza-FAD–General Acyl-CoA Dehydrogenase. A trace of solid NaB^3H_4 was added to 1.2 mL of a 10^{-4} M solution of 5-deaza-FAD (120 nmol) adjusted to pH 10.2 with K_2CO_3. Bleaching of the flavin was followed spectrally. After 40 min 10 μL of acetone was added to destroy excess reductant, and the pH was adjusted to neutrality with KH_2PO_4. The reduced deaza-FAD was then incubated for 20 min at 4 °C with 0.6 mL of 250 μM acyl-CoA dehydrogenase apoprotein (Mayer & Thorpe, 1981) and then applied to a Sephadex G-25 fine column (void volume 8 mL) equilibrated with 0.05 M phosphate buffer, pH 7.6. Elution with the same buffer yielded a protein fraction containing reduced 5-deaza-FAD–enzyme with a specific activity of approximately 9000 cpm/nmol.

Alternatively, 43 nmol of 5-deaza-FAD–enzyme in 1 mL of 0.05 M pyrophosphate, pH 9, was first treated with 5 nmol of methylenecyclopropylacetyl-CoA to inactivate any residual native (FAD) enzyme (Wenz et al., 1981) and then with a total of 0.6 mg of NaB^3H_4 (NEN, lot 1576-211, 8 mCi/mg). The reduction proceeded to ~85% completion, thus forming ~37 nmol of reduced 5-deaza enzyme. Residual borotritide was destroyed by adding 3 mg of sodium pyruvate, and the solution was chromatographed over a Sephadex G-25 fine column (void volume 11 mL) equilibrated with 0.05 M phosphate buffer, pH 7.6. The eluted enzyme fraction had a specific activity of ~30 000 cpm/nmol, and ~38 nmol of enzyme was recovered.

Reduction of Crotonyl-CoA with Tritiated, Reduced 5-Deaza-FAD–General Acyl-CoA Dehydrogenase. The reduced enzyme obtained by reconstitution of tritiated, reduced 5-deaza-FAD with apoenzyme (~8 nmol) was titrated (cf. Figure 2) with crotonyl-CoA (total ~8 nmol), then applied to a G-25 fine Sephadex column (void volume 6 mL) equilibrated with 0.05 M phosphate, pH 7.6, and eluted with the same buffer. The concentration of the enzyme in the eluate was estimated by its absorption at 390 nm [extinction coefficient 16 700 M^{-1} cm^{-1} (Thorpe & Massey, 1983)] and that of the CoA derivatives by the absorbance at 260 nm (ϵ ~ 16 000 M^{-1} cm^{-1}). Approximately 7 nmol of oxidized enzyme with a specific activity of 5100 cpm/nmol and 8 nmol of CoA derivatives (4400 cpm/nmol) (cf. Figure 3) were recovered.

In typical complementary expeiments, 16 nmol of enzyme (obtained by direct reduction of the oxidized 5-deaza-FAD–enzyme with NaB^3H_4) in 0.8 mL of buffer, pH 7.6, was titrated with 1.2 equiv of crotonyl-CoA. When the reaction mixture was chromatographed on Sephadex G-25, 27% of the applied counts were recovered in the protein fraction and the remaining 73% in the included volume. Denaturation of the protein fraction either by addition of 5% trichloroacetic acid or by heating to 100 °C for 2 min, and subsequent separation of the protein from the supernatant by centrifugation or by membrane filtration (exclusion volume 25 000 daltons), indicated that ~75% (±10% average of three experiments) of the radioactivity eluted in the protein fraction was covalently linked to the protein. The remainder was released to the supernatant.

The specific activity of the free 5-deaza-FAD–coenzyme was determined after heat denaturation (2 min at 100 °C) of the enzyme using the high-pressure liquid chromatography (HPLC) technique described by Wenz et al. (1981). When 6.5 nmol of enzyme was analyzed before reoxidation with crotonyl-CoA, 0.9 nmol of oxidized 5-deaza-FAD was obtained (5700 cpm/nmol; quantitated by fluorometric determination after hydrolysis to the FMN derivative with *Naja naja* venom) together with 5.5 nmol of reduced 5-deaza-FAD (20 000 cpm/nmol; quantitated by the absorbance at 310 nm; Stankovich & Massey, 1976). When the same analysis was applied to a sample (total 150 000 cpm) that had been reoxidized with crotonyl-CoA, 83 000 cpm were eluted with butyryl-CoA and 8500 cpm with oxidized 5-deaza-FAD (1825 cpm/nmol). Less than 5% (~4000 cpm) of the total eluted counts were found as nonreacted, reduced 5-deaza-FAD. Most of the difference between the radioactivity originally contained in the sample (150 000 cpm) and the total eluted from HPLC (95 000 cpm) was found to be bound covalently to the denatured protein (50 000 cpm). The latter was analyzed by centrifugation, washing of the precipitate, and digestion with Pronase before counting.

Release of Label from [3-^3H]Butyryl-CoA with Butyryl-CoA Dehydrogenase. Approximately 1.2 nmol of the labeled butyryl-CoA obtained from reoxidation of labeled, reduced 5-deaza-FAD–general acyl-CoA dehydrogenase (see Figure 3) was incubated aerobically with 0.1 nmol of butyryl-CoA dehydrogenase for 20 min. Product analysis was done by anion-exchange separation as described above (cf. also Table II).

Chemical Release of Label from Butyryl-CoA. These experiments were similar to those described by Murfin (1974). Aliquots (10 μL) of labeled butyryl-CoA (~2000 cpm, cf. Table II) in 50 μL of 12 N NaOH were sealed in Pyrex vials under nitrogen and heated at 110 °C for the times shown in Table II. The products were analyzed with 0.5-mL Dowex anion-exchange columns.

Results and Discussion

Kinetics and Stoichiometry of Incorporation of Solvent Protons in Butyryl-CoA Catalyzed by Butyryl-CoA Dehydrogenase. Gomes et al. (1981) have shown that butyryl-CoA dehydrogenase from *M. elsdenii* catalyzes the ex-

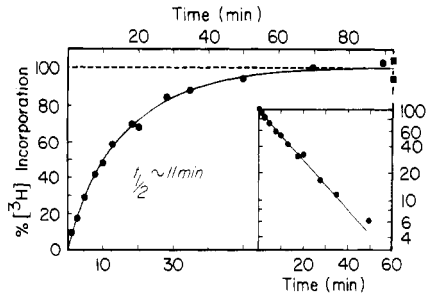


FIGURE 1: Kinetics of ^3H incorporation from solvent into the α- and β-positions of butyryl-CoA catalyzed by butyryl-CoA dehydrogenase. Butyryl-CoA (800 nmol) was incubated in 0.1 mL of tritiated water (0.05 M phosphate buffer, pH 7.6) under anaerobic conditions with *M. elsdenii* butyryl-CoA dehydrogenase (0.2 nmol). At the time intervals shown (●), aliquots were analyzed for incorpoation into butyryl-CoA. As the curve was constructed from several experiments, the incorporation of label has been normalized to 100%; an average analysis point at 100% contained approximately 4000 cpm. The solid squares (■) represent the incorporation found under similar conditions after 20 min, but using 10-fold more enzyme. The inset shows a semilogarithmic plot of the exchange process. Note that the incorporation proceeds monophasically for at least 3 half-lives.

change of the α- and β-protons of butyryl-CoA in tritiated water to approximately equal extents; however, the overall stoichiometry and kinetics of incorporation were not addressed specifically. We conider a combination of such data with kinetic parameters important for mechanistic interpretations, as exchange might arise by pathways alternative to the catalytic sequence. We have chosen an approach similar to that of Gomes et al. (1981) but have conducted these exchange experiments anaerobically (Figure 1). Under these conditions, using an efficient oxygen-scavenging system, exchange is first order over 3 half-lives (Figure 1). Four anaerobic incubations, starting with ^3H activities of 25–50 cpm/nmol of H_2O and 200–800 nmol of butyryl-CoA, gave a stoichiometry of 1.94 ± 0.10 hydrogens exchanged per substrate molecule (see Materials and Methods). However, when exchange studies similar to those shown in Figure 1 were performed aerobically as described by Gomes et al. (1981), an increase in tritium incorporation was followed by a decline reflecting the net oxidation of butyryl-CoA to crotonyl-CoA. Indeed, the bacterial butyryl-CoA dehydrogenase exhibits appreciable oxidase activity (Engel & Massey, 1971).

The NMR spectrum of the butyric acid obtained after exchange of the thio ester in ^2H_2O buffer is shown in Figure 2. The triplet of the butyric acid $-CH_3$ group has now reduced to a doublet (three protons). The sextet of the β-methylene group (1.45 ppm) and the triplet of the α-methylene group (2.08 ppm) collapse to broad signals (geminal interactions with ^2H) and now integrate to one proton each. The corresponding signals of normal butyric acid integrate to intensities in the ratio 3:2:2. The conclusion that both the α- and β-hydrogens undergo exchange is also fully supported by gas chromatographic–mass spectroscopic analysis of the same butyric acids. The mass of the molecular ions (90 and 88) and the masses of two of the major fragments (m/z = 61 and 60 and m/z = 29 and 28) for deuterated and normal butyric acid indicate a deuterium content >95%.

These data, together with those of Gomes et al. (1981), show clearly that both the α- and β-hydrogens are exchanged and, in particular, that both hydrogens are exchanged at the same rate. The rate of exchange has a half-time of 11 min under the conditions of Figure 1, and taking a tangent to the curve in Figure 1, this rate corresponds to approximately 300 substrate molecules exchanged min^{-1} (molecule of enzyme)$^{-1}$. Engel & Massey (1971) report a turnover rate of 200 mol of

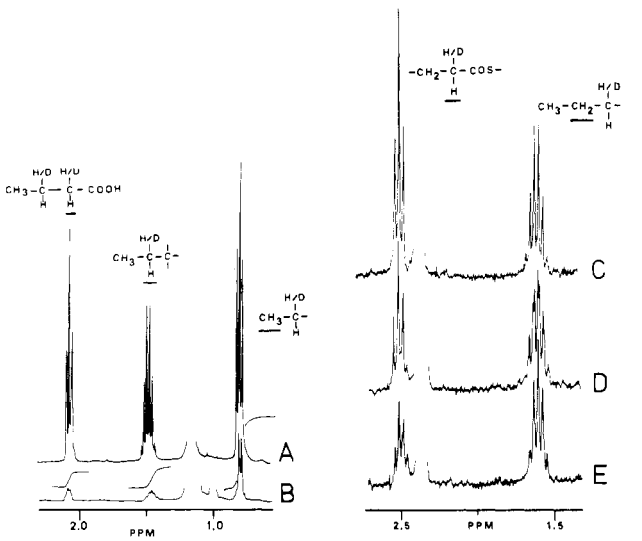
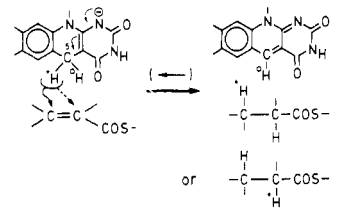


FIGURE 2: ^1H NMR determination of hydrogen exchange at the α- or β-positions of butyryl-CoA catalyzed by the enzymes general acyl-CoA dehydrogenase and butyryl-CoA dehydrogenase. (Left-hand side) Approximately 5 μmol of butyryl-CoA was incubated anaerobically in 0.05 M phosphate buffer in ^2H_2O (which had a hydrogen ion activity corresponding to pH 7.6), and in the presence of 10 nmol of *M. elsdenii* butyryl-CoA dehydrogenase. The exchange, and the workup, was carried out as detailed under Materials and Methods. Trace A shows the spectrum of free butyric acid for comparison and trace B shows that of the incubation sample, both in CCl_4. The lines between the traces represent the integration of trace B and show that the γ-, β-, and α-hydrogens are present in a ratio of 3:1:1. Note that the signal of the γ-CH_3 groups yields a doublet upon exchange of one β-hydrogen. (Right-hand side) In an analogous experiment, 5 mM butyryl-CoA was incubated at 25 °C in the same deuterated buffer with 9 μM pig kidney general acyl-CoA dehydrogenase, the spectra shown being recorded directly in the NMR tube. Trace C shows the resonances of the α- and β-hydrogens in the absence of enzyme. Traces D and E are the spectra of the same hydrogens 45 and 90 min, respectively, after addition of general acyl-CoA dehydrogenase. Note that the intensity of the signal of the α-hydrogen has decreased by 50% upon incubation. Concomitantly, the splitting patterns of the β-hydrogen, but not its intensity, are modified significantly [trace C (sextet) to trace E (quintet)]. Only the resonances relevant to the experiment are shown; signals originating from the CoA moiety have been deleted for clarity. Traces A and B were recorded with a Bruker 360-MHz instrument and traces C–E with a Bruker AM 250-MHz instrument.

substrate min^{-1} (mol of enzyme)$^{-1}$ for oxidation of butyryl-CoA for the same enzyme. Thus, the rate of exchange is similar to the rate of catalytic turnover. This also suggests that in this enzyme the exchange of the α- and β-hydrogens indeed occurs during the catalytic event.

Kinetics and Stoichiometry of Incorporation of Solvent Protons into Butyryl-CoA Catalyzed by General Acyl-CoA Dehydrogenase. In contrast to *M. elsdenii* butyryl-CoA dehydrogenase, general acyl-CoA dehydrogenase from pig kidney catalyzes the exchange of only the α-hydrogen of butyryl-CoA. This was demonstrated directly by following the NMR spectrum of butyryl-CoA in ^2H_2O after the addition of enzyme (see Figure 2). Trace C shows the spectrum of butyryl-CoA prior to exchange, in which the expected splitting pattern is observed. After a 90-min incubation (trace E), one of the two α-hydrogens was found to have exchanged, as shown by the decrease of the triplet intensity. At the same time, the sextet of the β-hydrogen evolves to a quintet, as required if only a single α-hydrogen were to undergo exchange. From the intermediate spectra, a half-time of exchange of ~45 min can be estimated. These observations are in agreement with a recent report by Ikeda et al. (1983) that rat liver acyl-CoA dehydrogenase catalyzes only the exchange of an α-hydrogen in octanoyl-CoA.

Scheme II



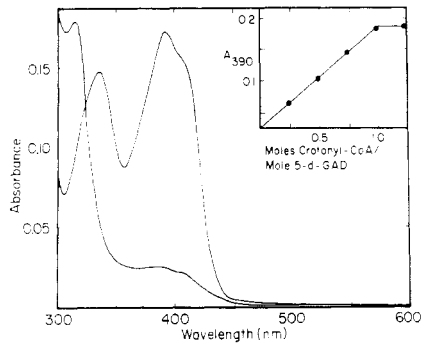


FIGURE 3: Titration of reduced 5-deaza-FAD–general acyl-CoA dehydrogenase with crotonyl-CoA. The enzyme was obtained either by reconstitution from apoenzyme and 1,5-dihydro-5-deaza-FAD formed by reduction of 5-deaza-FAD (Materials and Methods) or by direct reaction of 5-deaza-FAD–enzyme with sodium borohydride. The lower curve shows the spectrum of reduced 5-deaza-FAD–enzyme. The inset shows the absorbance changes at 390 nm upon addition of aliquots of crotonyl-CoA. The upper curve represents the spectrum obtained at the end of the titration; it is identical with that of oxidized 5-deaza-FAD–general acyl-CoA dehydrogenase.

Evidence for Transfer of ^3H from the Butyryl-CoA Substrate to the Flavin 5-Position. As mentioned in the introduction, depending on the reaction mechanism envisaged, either the α- of the β-hydrogen (or neither of them) might be transferred to the flavin during oxidation of the substrate. This problem cannot be addressed by using the normal prosthetic group since dihydroflavins exchange protons with solvent at position N(5). However, the use of 5-deazaflavins has allowed the demonstration of transfer of hydride equivalents from the bound substrate to the prosthetic group in a number of flavoenzymes [e.g., see Jorns & Hersh (1975), Averill et al, (1975), Spencer et al. (1976), Pompon & Lederer (1979), and Hersh & Walsh (1980)] since transfer occurs to the now nonexchangeable C(5)–position. In the present case, reduction of 5-deaza-FAD–acyl-CoA dehydrogenase by butyryl-CoA is thermodynamically unfavorable (Thorpe & Massey, 1983), and so the reaction was run in reverse (Scheme II). Figure 3 shows that reoxidation of the 1,5-dihydro-5-deaza-FAD–enzyme by crotonyl-CoA is essentially stoichiometric. The kinetics of this reaction are addressed later in this paper.

When the reoxidation was conducted starting with reduced [5-^3H]deaza-FAD–enzyme, in which the 5-deaza-FAD was first reduced with NaB^3H_4 and then reconstituted with apoprotein, two radioactive fractions eluted on a Sephadex G-25 column (Figure 4). The amount of ^3H included in the fractions eluting in the void volume (protein fraction) and that found in the small-molecules fraction were nearly equal. Oxidized 5-dFAD–general acyl-CoA dehydrogenase in the protein eluate and a CoA-derivative, butyryl-CoA (see below), were identified and quantitated in the two fractions by their absorption spectra. The specific activity was 5100 cpm/nmol for the enzyme and 4400 cpm/nmol for the CoA fractions. These results are as expected, since borotritide reduction of free 5-deazaflavin would yield two enantiomers at C(5): Upon reconstitution with apoprotein, only one of these species could

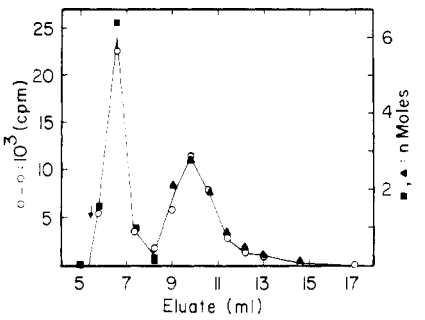


FIGURE 4: Gel filtration profile of the products obtained from reaction between reduced, 5-^3H-labeled deaza-FAD–enzyme and crotonyl-CoA. The reaction was carried out as described in the legend to Figure 3. After completion of the titration, separation of the products on a Sephadex G-25 column yielded the elution profile shown: (○) radioactivity in the eluted fractions; (■) protein-bound 5-deaza-FAD, estimated by the absorbance at 390 nm; (▲) CoA derivatives, estimated by the absorbance at 260 nm. The arrow indicates the void volume of the column.

transfer a tritium to crotonyl-CoA.

On the other hand, when reduction of crotonyl-CoA was carried out with enzyme obtained by direct reduction of 5-deaza-FAD–enzyme with NaB^3H_4, the counts found in the CoA ester fraction (by HPLC analysis) and those remaining with oxidized 5-deaza-FAD have a ratio of 91 to 9. The specific activity of the reduced 5-deaza-FAD employed for reduction was 19 800 cpm/nmol, and that of the oxidized 5-deaza-FAD recovered was ~1800 cpm/nmol. Thus, the two sets of data coincide very well and indicate that in this case ~90% of the label is transferred to the substrate during oxidation. The residual 10% presumably reflects nonspecific reduction by borohydride.

From the principle of microscopic reversibility, the reverse reaction, i.e., the transfer from butyryl-CoA to oxidized 5-deaza-FAD–general acyl-CoA dehydrogenase, must also occur by the same mechanism.

Demonstration That the β-Hydrogen of Butyryl-CoA Is Transferred to the Flavin. The analysis of label incorporated into the α- and β-positions of fatty acid has been performed by Gomes et al. (1981) by chemical degradation, and by Murfin (1974), taking advantage of the fact that only the α-protons and not the β-protons of fatty acids possess enough activation to undergo exchange with solvent at high pH. The latter method was adopted here due to the very small amounts of material available. Table II shows that with tritiated butyryl-CoA, obtained by solvent exchange with *M. elsdenii* butyryl-CoA dehydrogenase, which is expected to have label at both the α- and β-positions, approximately 50% of the label is sensitive to alkaline exchange. However, with the material obtained from reaction of reduced [5-^3H]deaza-FAD–general acyl-CoA dehydrogenase with crotonyl-CoA, the bulk of the label is retained. The deviation of ~10% from the ideal values of 50% and 100% retention for the two compounds is attributed to problems arising from the experimental conditions (heating of the sample at 110 °C in 12 N KOH for 40 h). The results in Table II establish two things. First, the proton which is transferred to the flavin in general acyl-CoA dehydrogenase is from the β-position of the substrate. Second, both medium- and short-chain acyl-CoA dehydrogenase have the same stereospecificity, since the β-proton of butyryl-CoA obtained from crotonyl-CoA oxidation of reduced 5-deaza-FAD–general acyl-CoA dehydrogenase is removed completely on incubation with bacterial butyryl-CoA dehydrogenase.

Comparison of Rates of Reoxidation of Native Reduced Enzyme and 5-Deaza-FADH$_2$–Enzyme with Crotonyl-CoA and Deuterium Isotope Effects. Reoxidation of 6.9 μM re-

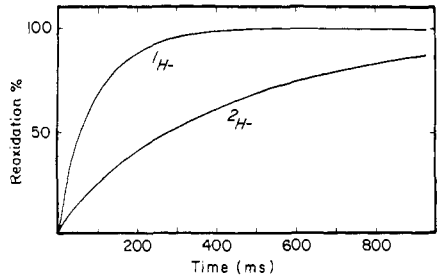
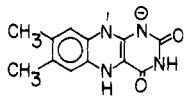
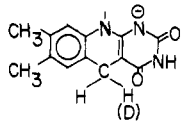
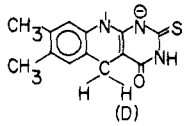


FIGURE 5: Reoxidation of 5-deaza-$FADH_2$- and [5-^{2}H]deaza-$FADH_2$-general acyl-CoA dehydrogenase by crotonyl-CoA. Apoprotein reconstituted with 5-deaza-FAD was reduced with either $NaBH_4$ or NaB^2H_4 and dialyzed vs. 0.05 M phosphate, pH 7.6. These enzyme samples were reacted at 25 °C with various concentrations of crotonyl-CoA in the same buffer. The absorbance increase at 390 nm was followed in a stopped-flow spectrophotometer. The results shown are for a 50 μM final concentration of crotonyl-CoA with an enzyme concentration of 6.5–6.9 μM for the two forms. Similar monophasic reaction curves were found at all wavelengths studied (320, 350, 400, 420, and 430 nm).

Table III: Rates of Reduction of Crotonyl-CoA by Different Reduced Flavin Forms of General Acyl-CoA Dehydrogenase

structure of reduced flavin in enzyme	k_{obsd} (s^{-1}) ^{1}H	^{2}H	isotope effect	redox potential (mV)[a]
	2.6			−210[b]
 (D)	11.1	2.2	5.0	−273[c] to −311[d]
 (D)	0.0053	0.0014	3.8	−195 to −230[e]

[a] Redox potential at pH 7.0 for the two-electron couples taken from the indicated references. [b] Draper & Ingraham (1968). [c] Stankovich & Massey (1976). [d] Walsh et al. (1978). [e] Estimated; see text.

duced 5-deaza-FAD–general acyl-CoA dehydrogenase was followed with a stopped-flow spectrophotometer under aerobic conditions in 0.05 M phosphate, pH 7.6 at 25 °C. Enzyme samples were prepared by reduction of the oxidized 5-deaza-FAD–protein with either NaB^1H_4 or NaB^2H_4, followed by dialysis vs. 0.05 M phosphate buffer. In both cases, reoxidation is smoothly monophasic and first order (see Figure 5) with no significant difference in k_{obsd} for each form over the range of 20–125 μM crotonyl-CoA. These results indicate a tight binding of crotonyl-CoA to the reduced enzyme ($K_d < 3$ μM) prior to reoxidation. The rates of reoxidation with 5-H- and 5-^{2}H-reduced 5-deaza enzyme are shown in Table III.

In companion experiments with native enzyme photoreduced with ethylenediaminetetraacetic acid (EDTA) and deazaflavin (Massey & Hemmerich, 1978) and reoxidized with crotonyl-CoA (20–125 μM) under anaerobic conditions, the observed kinetics were again independent of crotonyl-CoA concentration. In the dead time of the apparatus (<3 ms), an intermediate with long-wavelength absorbance maximal at 570 nm was formed. This decayed monophasically to oxidized enzyme with a k_{obsd} of 2.6 s^{-1}. The intermediate had spectral characteristics qualitatively and quantitatively similar to those found on titration of 8-chloro-FAD–enzyme by butyryl-CoA (Thorpe & Massey, 1983) and presumably is a charge-transfer complex between reduced enzyme and crotonyl-CoA.

These results may be interpreted by reference to the following equilibria (Beinert & Page, 1957; Thorpe et al., 1979):

$$EFl_{ox} + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EFl_{ox}S \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} EFl_{red}P \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} EFl_{red} + P$$

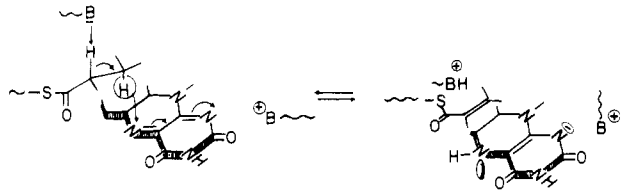
$$EFl_{ox} + P \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} EFl_{ox}P$$

Starting with reduced enzyme and crotonyl-CoA, the results with both native enzyme and 5-deaza-FAD–enzyme show that the primary association is established rapidly, with a K_d (k_3/k_{-3}) in the range of micromolar or less. The value of k_{-2} determined for native enzyme (Table III) is in good agreement with the value of ~4 s^{-1} obtained by computer-simulated data fitting of the reaction of native enzyme with butyryl-CoA (B. Pohl and S. Ghisla, unpublished results). These same data with native enzyme yield a value for k_2 of 3 s^{-1}. With 5-deaza-FAD–enzyme, the value of k_2 is ~0, since this enzyme is not reduced significantly by butyryl-CoA, presumably because of the lower redox potential of 5-deaza-FAD compared to FAD (Table III). While butyryl-CoA clearly binds to oxidized 5-deaza-FAD–enzyme, the spectral perturbations are small and do not permit an accurate estimation of K_d (~10–100 μM). With crotonyl-CoA, the spectral perturbations are more marked with a K_d (k_{-4}/k_4) of ~20 μM at pH 7.6, 4 °C. This value compares favorably with the value for binding of crotonyl-CoA to native enzyme under the same conditions (18–25 μM; this paper, data not shown). Hence, it is clear that the 5-deaza-FAD–enzyme, apart from the vanishingly small value of k_2, exhibits behavior similar to that of the native enzyme. We attempted to circumvent this unfavorable thermodynamic barrier by using 2-thio-5-deaza-FAD instead of 5-deaza-FAD (see structures in Table III). The redox potential of the former should be raised by a similar extent as that of 2-thio-FAD compared with normal FAD, and thus be in the range of −195 to −230 mV. Holoenzyme formed by reconstitution with 2-thio-5-deaza-FAD, however, was not reduced by butyryl-CoA. It should be noted that the native enzyme is only about 75% reduced at saturating levels of butyryl-CoA (Thorpe et al., 1979; B. Pohl and S. Ghisla, unpublished results), suggesting that the redox potential of the enzyme-bound 2-thio-5-deaza-FAD probably is still too low for reduction to occur with butyryl-CoA. In agreement with this assumption, reduced 2-thio-5-deaza-FAD–enzyme is completely reoxidized by a small excess of crotonyl-CoA. Reoxidation is monophasic but occurs at a much slower rate compared with native reduced enzyme or with 5-deaza-$FADH_2$–enzyme (Table III).

The finding of a $^1H/^2H$ isotope effect of 5 in the reoxidation of reduced 5- and [5-^{2}H]deaza-$FADH_2$–enzyme by crotonyl-CoA implies that flavin C(5)H bond rupture is rate limiting in this reaction. Although the reverse reaction cannot be observed because of the thermodynamic barrier imposed by the low redox potential of 5-deazaflavin, the law of microscopic reversibility dictates that a similar rate-limiting transfer of H from substrate to flavin must occur. In fact, an isotope effect ($^1H/^2H$) of ~7 has been observed in the reduction of native enzyme with[β-$^2H_2$]butyryl-CoA (B. Pohl and S. Ghisla, unpublished results).

The kinetics of reoxidation of 5- and [5-^{2}H]deaza-$FADH_2$–enzyme also indicate that the reduction of 5-deaza-FAD–enzyme by $NaBH_4$ is largely stereospecific for one flavin face and that the hydrogen transferred during borohydride reduction is the same one that is removed on reoxidation with crotonyl-CoA. This conclusion follows not only from the

Scheme III



magnitude of the isotope effect but also from the fact that the deuterated enzyme is reoxidized monophasically (cf. Figure 5). If the NaB^2H_4 reduction had occurred randomly at both flavin faces, the reoxidation would have occurred half in a fast and half in a slow phase.

Conclusions

In addressing some of the questions raised in the introduction, we have made use of enzyme derivatives substituted with 5-deaza-FAD. Several authors have emphasized the need for caution when comparing results obtained with 5-deazaflavins with normal flavin, since the former possess analogies with nicotinamides as well as flavin (Farng & Bruice, 1983; Hemmerich et al., 1977). The validity of a mechanistic conclusion is made more certain when results from static data can be correlated with kinetic parameters. Thus, when chemically different flavins react with the same substrate at comparable rates, the conclusion appears likely that they also react via the same mechanism. It would appear unlikely that the orientation of the reaction partners within the active center would allow for two mechanistically different sets of reactions to occur at comparable rates. In our instance, for a hydride mechanism to occur, the β-hydrogen of the substrate is required to be sandwiched between the substrate carbon chain and the flavin plane in the projection of the flavin position 5π-acceptor orbital (Scheme III). As the reaction involves removal of a trans pair of hydrogens, the base abstracting the α-proton must be positioned on the opposite "face" of the substrate (Scheme III). In agreement with the conclusions of Farng & Bruice (1983) from the chemical system, we propose that α,β-dehydrogenation of acyl-CoA substrates occurs by a carbanion–hydride transfer mechanism, as shown in Scheme III. A carbanion-initiated mechanism is also consistent with other results as reviewed in the introduction. The differences in rates of exchange between the α- and β-hydrogens of substrate observed with different enzymes clearly indicate that the active centers of these enzymes have different accessibilities of solvent and protons. A notable difference between the bacterial enzyme and mammalian acyl-CoA dehydrogenases is the considerable oxidase activity of the former (Engel & Massey, 1971). While *Megasphera elsdenii* is a strict anaerobe, mammalian enzymes transfer reducing equivalents efficiently to the natural acceptor electron-transferring flavoprotein in an aerobic environment. The different oxygen reactivity might thus simply reflect a genetic adaptation of mammalian enzymes to prevent access of oxygen to the flavin, this shielding in turn also affecting the access of solvent protons to the active site. The present data are compatible with a catalytic process in which the carbanionic species might have a finite lifetime and thus undergo hydrogen exchange, as well as with exchange occurring at the level of the reduced enzyme–crotonyl-CoA complex. In the latter case, however, only the base serving the α-proton abstraction would be exposed to solvent, while the flavin N(5)–H function would be shielded by the protein and the bound product molecule (Scheme III).

The finding that BH_4^- and substrate react on the same face of the flavin is of considerable interest and could be of practical importance in determination of the absolute stereospecificity of flavin–enzyme reactions. The question of which flavin face reacts in enzymatic reactions has been addressed by Walsh et al. (Spencer et al., 1976) and by our groups (Ghisla & Massey, 1980; Ghisla et al., 1980) with other enzymes. The absolute stereochemistry has, however, so far eluded determination. The accessibility of specific labeling of deazaflavin position 5 when bound to general acyl-CoA dehydrogenase apoenzyme, and the potential of transferring the specifically labeled 5-deazaflavin to enzymes of known active center structure, such as glutathione reductase (Schulz et al., 1982), might make it possible to determine the stereochemistry of the whole group of acyl-CoA-oxidizing enzymes, and possibly other flavoproteins.

Acknowledgments

We thank Dr. Lawrence G. Schopfer for carrying out the stopped-flow experiments, Dr. Oksana Lockridge for HPLC analyses, Frank Parker and Neil Dando for NMR analyses, and Dr. Thomas Kuster for mass spectroscopic measurements.

Registry No. 5-Deaza-FAD, 57818-88-9; butyryl-CoA, 2140-48-9; crotonyl-CoA, 992-67-6; acyl-CoA dehydrogenase, 9027-65-0; butyryl-CoA dehydrogenase, 9027-88-7; D_2, 7782-39-0; T_2, 10028-17-8.

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NMR Studies of the Backbone Protons and Secondary Structure of Pentapeptide and Heptapeptide Substrates Bound to Bovine Heart Protein Kinase†

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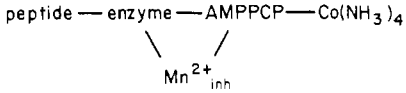
ABSTRACT: The conformations of enzyme-bound pentapeptide (Arg-Arg-Ala-Ser-Leu) and heptapeptide (Leu-Arg-Arg-Ala-Ser-Leu-Gly) substrates of protein kinase have been studied by NMR in quaternary complexes of the type



Paramagnetic effects of Mn^{2+} bound at the inhibitory site of the catalytic subunit on the longitudinal relaxation rates of backbone C_α protons, as well as on side-chain protons of the bound pentapeptide and heptapeptide substrates, have been used to determine Mn^{2+} to proton distances which range from 8.2 to 12.4 Å. A combination of the paramagnetic probe–T_1 method with the Redfield 2–1–4–1–2 pulse sequence for suppression of the water signal has been used to measure distances from Mn^{2+} to all of the backbone amide (NH) protons of the bound pentapeptide and heptapeptide substrates, which range from 6.8 to 11.1 Å. Paramagnetic effects on the transverse relaxation rates yield rate constants for peptide exchange, indicating that the complexes studied by NMR dissociate rapidly enough to participate in catalysis. Model-building studies based on the Mn^{2+}–proton distances, as well as on previously determined distances from Cr^{3+}-AMPPCP to side-chain protons [Granot, J., Mildvan, A. S., Bramson, H. N., & Kaiser, E. T. (1981) *Biochemistry 20*, 602], rule out α-helical, β-sheet, β-bulge, and all possible β-turn conformations within the bound pentapeptide and heptapeptide substrates. The distances are fit only by extended coil conformations for the bound peptide substrates with a minor difference between the pentapeptides and heptapeptides in the ϕ torsional angle at Arg_3C_α and in ψ at Arg_2C_α. An extended coil conformation, which minimizes the number of interactions within the substrate, would facilitate enzyme–substrate interaction and could thereby contribute to the specificity of protein kinase.

Adenosine cyclic 3′,5′-monophosphate (cAMP)[1] dependent protein kinase plays a major role in the regulation of certain enzymes through selective phosphorylations (Krebs & Beavo, 1979). Dissociation of the regulatory subunit from the catalytic subunit, a process mediated by cAMP, has been shown to activate the catalytic subunit by allowing the binding of peptide or protein substrates (Granot et al., 1980a).

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[1] Abbreviations: cAMP, adenosine cyclic 3′,5′-phosphate; Co$(NH_3)_4$ATP, tetraammine(adenosine triphosphate-P^β,P^γ)cobalt(III); Co$(NH_3)_4$AMPPCP, tetraammine(adenosine β,γ-methylenetriphosphate-P^β,P^γ)cobalt(III); CrAMPPCP β,γ-bidentate, Cr^{3+}-β,γ-methylene-ATP; DTT, dithiothreitol; DSS, sodium 4,4-dimethyl-4-silapentane-1-sulfonate; MES, 2-(*N*-morpholino)ethanesulfonic acid; Tris, tris(hydroxymethyl)methylamine; MOPS, 3-(*N*-morpholino)propanesulfonic acid; pH*, meter reading in ^2H_2O; Ser-pentapeptide, Arg-Arg-Ala-Ser-Leu; Ser-heptapeptide, Leu-Arg-Arg-Ala-Ser-Leu-Gly; Tyr-heptapeptide, Leu-Arg-Arg-Tyr-Ser-Leu-Gly; Ala-heptapeptide, Leu-Arg-Arg-Ala-Ala-Leu-Gly; A/D, analogue to digital; FID, free induction decay.

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