The Coenzyme Analogue Adenosine 5-Diphosphoribose Displaces FAD in the Active Site of *p*-Hydroxybenzoate Hydroxylase. An X-ray Crystallographic Investigation†

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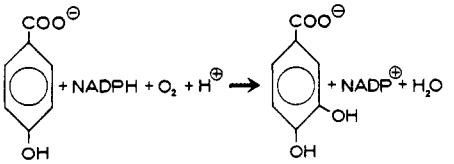
ABSTRACT: *p*-Hydroxybenzoate hydroxylase (PHBH) is an NADPH-dependent enzyme. To locate the NADPH binding site, the enzyme was crystallized under anaerobic conditions in the presence of the substrate *p*-hydroxybenzoate, the coenzyme analogue adenosine 5-diphosphoribose (ADPR), and sodium dithionite. This yielded colorless crystals that were suitable for X-ray analysis. Diffraction data were collected up to 2.7-Å resolution. A difference Fourier between data from these colorless crystals and data from yellow crystals of the enzyme–substrate complex showed that in the colorless crystals the flavin ring was absent. The adenosine 5′-diphosphate moiety, which is the common part between FAD and ADPR, was still present. After restrained least-squares refinement of the enzyme–substrate complex with the riboflavin omitted from the model, additional electron density appeared near the pyrophosphate, which indicated the presence of an ADPR molecule in the FAD binding site of PHBH. The complete ADPR molecule was fitted to the electron density, and subsequent least-squares refinement resulted in a final *R* factor of 16.8%. Replacement of bound FAD by ADPR was confirmed by equilibrium dialysis, where it was shown that ADPR can effectively remove FAD from the enzyme under mild conditions in 0.1 M potassium phosphate buffer, pH 8.0. The empty pocket left by the flavin ring is filled by solvent, leaving the architecture of the active site and the binding of the substrate largely unaffected.

p-Hydroxybenzoate hydroxylase (PHBH)[1] (EC 1.14.13.2) from *Pseudomonas fluorescens* is a flavin-containing monooxygenase with a molecular mass of 44 000. It incorporates one hydroxyl group in the substrate *p*-hydroxybenzoate in the reaction



The splitting of the oxygen into a hydroxyl group and a water molecule is believed to proceed via a flavin 4a-hydroxy peroxide intermediate (Entsch et al., 1976). Actually PHBH catalyzes two reactions; one is the already mentioned incorporation of oxygen into pOHB, and the other reaction is the reduction of the enzyme-bound flavin by the coenzyme NADPH, which can only proceed efficiently when substrate is bound (Husain & Massey, 1979; Müller, 1985). The X-ray structures of the enzyme in complex both with its substrate (pOHB) and with its product (3,4-diOHB) are described in detail (Wierenga et al., 1979; Weijer et al., 1983; Wierenga, 1986; Schreuder et al., 1988). Consequences of this work for the mechanism of hydroxylation have been reported by Schreuder et al. (1987, 1988).

On the other hand, structural studies on the reduction by NADPH are hampered by the fact that until now experiments to reveal the NADPH binding site failed. Attempts to predict a plausible binding site for NADPH by recognition of a common structural motif such as the nucleotide binding fold have been made. In the X-ray structures of many dinucleotide binding proteins, dinucleotides bind preferentially at the carboxyl edge of a parallel β-sheet with their pyrophosphate group near the N terminus of at least one α-helix (Rao & Rossmann, 1973; Wierenga et al., 1985). The common chain topology is $\beta\alpha\beta\alpha\beta$. In the X-ray structure of the flavoprotein glutathione reductase, such folds were recognized, one in the FAD binding domain, the other in the NADPH binding domain (Schulz et al., 1982).

In PHBH only the FAD binding domain shows the nucleotide binding fold (Wierenga et al., 1983). This observation suggests that the NADPH binding mode with PHBH is different from what is commonly observed for dinucleotides. Possibly PHBH relies on a quite different type of nucleotide binding such as has been observed for staphylococcal nuclease (Arnone, 1971) or the AMP site in phosphorylase *b* (Sygush et al., 1977), where arginines play a major role in accommodating the phosphate groups of the nucleotides.

Taking into account the accessibility of the flavin ring which has to be reduced by the nicotinamide and the position of the arginines in PHBH, a possible binding mode for NADPH has been proposed (Wierenga et al., 1982).

To locate the NADPH binding, a great number of soaking experiments have been performed. Crystals of the enzyme–substrate complex were soaked in high concentrations of coenzyme or well-defined fragments thereof. At the same time cocrystallization experiments were performed.

† The coordinates in this paper have been submitted to the Brookhaven Protein Data Bank.

[1] Abbreviations: PHBH, *p*-hydroxybenzoate hydroxylase (EC 1.14.13.2); Tris, 2-amino-2-(hydroxymethyl)propane-1,3-diol; EDTA, ethyldiaminetetraacetate; FAD, flavin adenine dinucleotide; pOHB, *p*-hydroxybenzoate; GSH, reduced glutathione; 3,4-diOHB, 3,4-dihydroxybenzoate; ADPR, adenosine 5-diphosphoribose; ADP, adenosine 5′-diphosphate; ADPRP, adenosine 2-monophospho-5-diphosphoribose; GDP, guanosine 5′-diphosphate; NADPH, reduced nicotinamide adenine dinucleotide phosphate.

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In one of these cocrystallization experiments, which were performed under anaerobic conditions, colorless single crystals grew from the PHBH substrate complex in the presence of the reducing agent sodium dithionite and the nucleotide adenosine 5-diphosphoribose (ADPR). Because these crystals were suitable for X-ray analysis, we collected data to 2.7-Å resolution. In this paper we describe the interaction of ADPR with PHBH.

Materials and Methods

Equilibrium Dialysis. Equilibrium dialysis experiments with ADPR were performed in 0.1 M potassium phosphate buffer, pH 7.5, at 4 °C. The PHBH concentration was determined from A_{280} measurement using A (1 mg/mL, 1 cm) = 2.63. The enzyme activity was assayed according to the procedure of Howell et al. (1972).

Crystallization. Isolation and purification of the PHBH that was used in the crystallization trails was performed according to the method described by Müller et al. (1979). Instead of a Sephadex–cibacron blue column we used a commercially available blue Sepharose Cl-6B column (Pharmacia). After the affinity chromatography step, the protein was applied to a Sephacryl S300 column that had been equilibrated with 0.1 M potassium phosphate buffer, pH 6.7.

Only the protein that eluted with the same retention time as bovine serum albumin was used for crystallization. It was shown that this fraction contains only PHBH dimers (Laan et al., 1989). A protein solution was prepared that contained 7.2 mg/mL PHBH dimers in 100 mM potassium phosphate buffer, pH 7.5, with 1 mM pOHB, 0.02 mM FAD, 0.15 mM EDTA, 0.10 mM GSH, and 60 mM ADPR. As precipitant we used a 70% ammonium sulfate solution in 100 mM potassium phosphate buffer, pH 7.5, with 1 mM pOHB, 0.02 mM FAD, 0.15 mM EDTA, and 0.10 mM GSH. A freshly prepared 1.0 M sodium dithionite ($Na_2S_2O_4$) stock solution in deaerated potassium phosphate buffer was added to both the protein and the ammonium sulfate solution to a final concentration of 120 mM. The pH of the solution was not corrected after the addition of dithionite.

Subsequently protein and ammonium sulfate solution (both 10 μL) were layered on top of each other in a melting point capillary as has been described before (Drenth et al., 1975; Laan et al., 1989). The capillaries were closed airtight by melting the open ends and stored at 4 °C in an oven. The reaction of dithionite with oxygen is expected to create an anaerobic atmosphere in the closed capillary. After 2 weeks, the temperature was slowly raised to 20 °C over a period of 2 weeks. No crystals were detected, and therefore the temperature was further raised to 32 °C during the next 2 weeks. Colorless crystals were observed after this period.

Data Collection. To mount the crystals, which had been grown in the presence of substrate, ADPR, and dithionite, special care was taken to avoid contact of the crystals with oxygen. The crystals were mounted in a closed glass vessel (height 3.0 cm, diameter 6.5 cm) which was placed under a microscope to follow manipulation of the crystal. One inlet of the vessel was connected to a nitrogen system to deoxygenate the vessel and to allow flushing with nitrogen during the mounting procedure. Through a second inlet a crystal could be transferred from a melting point capillary directly into the deoxygenated X-ray capillary inside the mounting vessel. All solutions through which the transfer of the crystal took place had been thoroughly deoxygenated just before the experiment. The artificial mother liquor contained 39% ammonium sulfate in 100 mM potassium phosphate, pH 7.5, with 1 mM pOHB, 0.01 mM FAD, 0.15 mM EDTA, 0.10 mM GSH, and 30 mM ADPR. The mother liquor was deaerated by flushing with nitrogen. Just before use the deaerated sodium dithionite stock solution was added to the mother liquor to a final concentration of 120 mM without any pH correction.

Preliminary precession photographs from anaerobically mounted colorless crystals show that the crystals have the same space group ($C222_1$) as the yellow crystals of the oxidized PHBH·FAD·pOHB complex, which were reported by Drenth et al. (1975). Compared to these crystals, the colorless crystals depict on zero-layer diffraction photographs certain differences in the diffraction pattern. Refinement of the cell parameters yielded 71.7, 146.4, and 89.2 Å for a, b, and c axes, respectively. In the native PHBH·FAD·pOHB crystals these values are 71.5, 145.8, and 88.2 Å (Drenth et al., 1975; Wierenga et al., 1979).

Oscillation data were collected on films (AGFA-D10) with an Enraf-Nonius oscillation camera mounted on an Elliott GX-6 rotating anode tube operating at 35 kV and 35 mA. A graphite monochromator from Robert Huber Diffraktionstechnik was used at a reflection angle of 13° to obtain Cu Kα radiation. Films were digitized on a Scandig microdensitometer (Joyce-Loebl) and processed by the OSCIL package, which is based on the Munich system (Schwager et al., 1975). In total 39 film packs, collected from four crystals, were processed, yielding 19 604 fully recorded reflections with an internal R_{sym} of 6.6% on F ($R_{sym} = \sum|F_i - \bar{F}|/\sum\bar{F}$). The final data set contained 9857 unique reflections and is 70% complete up to 2.7 Å.

Because a plot of the mean intensity sampled as a function of the resolution in a cone around each of the crystallographic axes showed that the fall off is anisotropic, the PHBH·pOHB·ADPR·dithio data were scaled to the native data by a least-square fitting procedure using one overall scale factor and six anisotropic B factors. The final R factor [$R = \sum|F_1 - F_2|/\sum(F_1 + F_2)$] between the two scaled data sets was 22.30% in F for 8275 common reflections. Subsequently, a difference Fourier was calculated between the data sets. The phases were calculated from the highly refined 1.9-Å native PHBH·FAD·pOHB structure (Schreuder et al., unpublished results).

In a preliminary $2F_o - F_c$ electron density map, calculated with phases derived from the refined PHBH·FAD·pOHB model, clear electron density was present for the adenosine and pyrophosphate moieties of FAD, but beyond the second phosphate no clear density was present for the ribitol chain and isoalloxazine ring. However, because the cell parameters of the ADPR–dithionite-grown crystals are slightly different from those of the native PHBH·FAD·pOHB complex, anisomorphism could obscure our results. Therfore, the position and the orientation of the complete PHBH molecule was optimized by constrained least-squares refinement using the program CORELS (Sussman et al., 1977).

Starting with the model of the native enzyme–substrate complex, we performed eight rounds of rigid body refinement using a randomly selected subset of 20% of the reflections between 10 and 4.0 Å. The R factor dropped from 33.6 to 26.0%. Subsequently, the model was divided into six rigid parts: the three domains and three long-chain excursions, which extend from the domains as clips keeping the multidomain PHBH molecule tighly together (Schreuder et al., 1988).

In a further eight rounds of CORELS refinement the R factor decreased only by 1%, which indicates that there are no large internal rearrangements. After CORELS, only an ADP moiety was added to the model. Eleven cycles of restrained least-

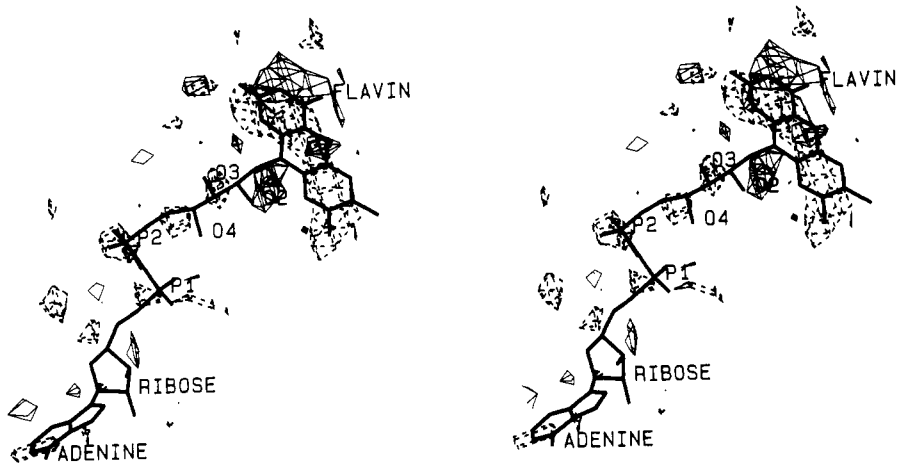

FIGURE 1: Stereoview of the difference electron density map $[(F_o - F_N) \exp(i\alpha_c)]$ around the FAD cofactor in the native PHBH substrate complex. F_o, observed structure factors from the PHBH crystals grown in the presence of ADPR and sodium dithionite; F_N, observed structure factor of the native PHBH·FAD·pOHB complex; phases calculated from the PHBH·FAD·pOHB model (R = 15.6%, 8). Negative contours are indicated by broken lines, positive contours by solid lines. The map was contoured at the 2.5σ level.

squares refinement (Hendrickson & Konnert, 1980a,b) without any solvent molecules yielded a crystallographic R factor of 18.9%. The 300 highest peaks in a $F_o - F_c$ difference map were examined, and 56 peaks were assigned to be solvent molecules. A blob of density had appeared near the pyrophosphate group in which a ribose could be fitted, which formed together with the ADP moiety the ADPR molecule. After six cycles of least-squares refinement, another four solvent molecules were located in the active site. On the basis of a $F_o - F_c'$ difference Fourier where the ADPR molecule had been left out from the calculation of F_c', the ADPR molecule was manually adjusted on an Evans and Sutherland PS390 graphics system running FRODO software (Jones, 1978). After three further cycles of restrained least-squares refinement, a final R factor of 16.8% was obtained for 8836 reflections between 2.7 and 6.0 Å. The results of this refinement are summarized in Table I. The atomic coordinates of this structure have been submitted to the Protein Data Bank at Brookhaven National Laboratories.

Table I: Final Refinement Parameters and Results[a]

	model	target σ
R factor[b]	16.8	
distances		
bond distances	0.015	0.020
angle distances	0.035	0.030
planar $^1/_4$ distances	0.037	0.040
planes	0.011	0.020
chiral volumes	0.175	0.150
nonbonded contacts		
single torsion contacts	0.171	0.200
multiple torsion contacts	0.194	0.200
possible hydrogen bonds	0.186	0.200
thermal parameter correlations		
main-chain bond	2.867	4.000
main-chain angle	3.957	4.000
side-chain bond	6.749	6.000
side-chain angle	8.773	9.000

[a] Model values are root mean square differences from ideal values (in Å for distances, in Å^2 for thermal parameter correlations, and in Å^3 for chiral volumes. Target σ is the inverse square root of the least-squares weight used for the parameter listed as described by Hendrickson and Konnert (1980a,b). [b] $R = \sum||F_{obs}| - |F_{calc}||/\sum|F_{obs}|$ (%).

RESULTS

Difference Fourier with Native PHBH. At first the absence of the characteristic yellow color of the flavin in crystals that were grown in the presence of substrate, ADPR, and sodium dithionite was ascribed to reduction of the flavin by dithionite. Therefore, the crystals were carefully mounted, avoiding any contact with air oxygen. However, the difference Fourier between data collected with the colorless ADPR–dithionite crystals and the native data collected from bright yellow crystals of the PHBH·FAD·pOHB complex (Wierenga et al., 1979) gave unexpected results as is shown in Figure 1.

The major features in the difference map are located around the flavin ring with extremes of -6σ and $+5\sigma$. Negative difference density around the flavin ring without compensating positive density nearby strongly suggests that the flavin ring is absent. Around the ribityl chain and the P2 phosphate the interpretation is less straightforward because positive as well as negative difference density is present. Around the adenosine diphosphate moiety only minor differences are observed, which indicates that this part of the FAD is still present. Only near the P2 phosphate is weak, negative difference density present. However, in the difference map between PHBH·pOHB·ADPR·dithio data and calculated structure factors from a PHBH model that contains only the adenosine monophosphate part of the FAD, the highest peak was found at the position of P2, which indicates this phosphate is still present. So we suppose that the negative density near P2 is due to a shift in position of the phosphate or to a change in mobility.

Beyond the P2 phosphate the map is less clear. Therefore, a refinement procedure was carried out, and in addition the effect of ADPR on PHBH in solution was further analyzed by equilibrium dialysis.

Effect of ADPR and Other Nucleotides on PHBH in Solution. Absence of the flavin implicates that the crystals should remain colorless when dithionite is removed and oxygen allowed. When crystals were soaked in aerated mother liquor without ADPR and dithionite, they stayed colorless and did not regain the yellow color of oxidized flavin. In contrast, when native yellow crystals are soaked with dithionite alone, they become colorless, but the yellow color is readily restored after removal of the reductant. This shows that reduction of the flavin by dithionite alone is a reversible process that can occur in the crystals. Soaking of native crystals in ADPR alone did not have any effect, neither on the color nor on the diffraction pattern of the soaked crystals. However, with PHBH in solution we observed an unexpected large effect of ADPR: the activity of the enzyme was completely blocked after prior addition of ADPR! Full activity could be restored by the addition of FAD, but not by the addition of NADPH. To study this phenomenon more in detail a fixed quantity of

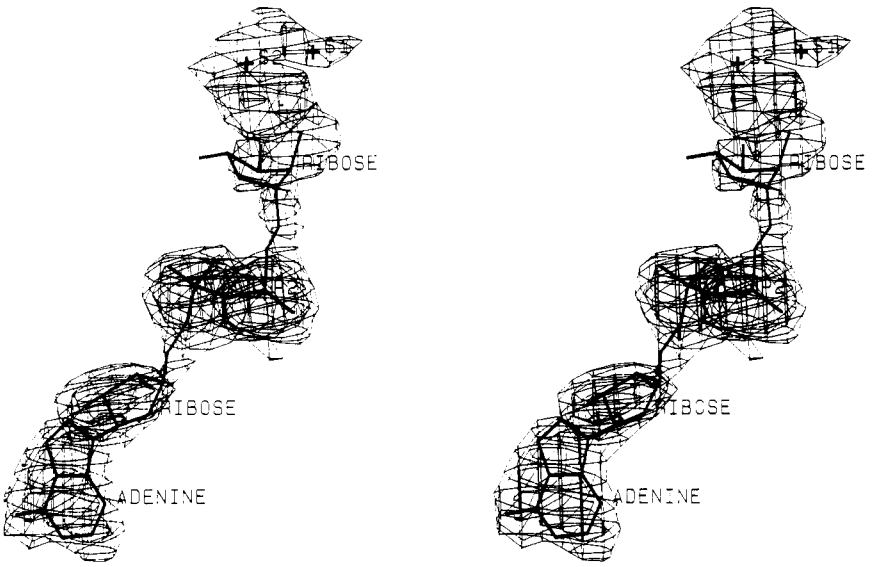

FIGURE 2: Stereoview of the $(F_o - F_c')$ exp $(i\alpha_c')$ difference Fourier, where the complete ADPR molecule has been left out from the F_c', $i\alpha_c'$ calculation. A σA weighting (Read, 1986) has been applied to the map coefficients. Also shown is the final model of the ADPR molecule and some bound solvent molecules, denoted by crosses. The map has been contoured at the 3.5σ level.

Table II: Equilibrium Dialysis of PHBH against ADPR[a]

ADPR (mM)	FAD content (%)	activity as percentage of original activity
0.05	84	90
0.11	78	75
0.20	59	63
0.44	53	53
0.60	33	42

[a] The protein (1 mL, A_{280}/A_{450} = 9.2, 2 mg/mL) was dialyzed against 25 mL of ADPR for 48 h (at 4 °C). Then the activity was determined in 0.1 M potassium pohsphate buffer, pH 8.0, without EDTA and FAD. The activity was calculated as the percentage of the activity of enzyme that was dialyzed against buffer. The FAD content was calculated from the A_{280}/A_{450} ratio, taking a A_{280}/A_{450} ratio of 9.2 for 100% FAD.

Table III: Effect of Various Nucleotides on the Binding of FAD in PHBH Determined by Equilibrium Dialysis[a]

	2 days	4 days	8 days
NAD + pOHB	9.73	11.25	12.4
NAD	9.58	10.33	14.1
ADP + pOHB	10.30	11.40	16.1
ADP	9.55	11.13	15.8
ADPR + pOHB	17.27	26.64	85.0
ADPR	17.00	28.80	
ADPRP	8.90	9.42	10.70
GDP	8.96	9.22	9.85
blank	9.12	9.22	10.10

[a] The conditions were the same as given in Table I. The table shows the E_{280}/E_{450} ratios after 2, 4, and 8 days of dialysis. Concentration of the nucleotides was 0.5 mM in each case; the pOHB concentration was 1 mM.

enzyme was dialyzed against increasing ADPR concentrations. The absorption at 450 nm of the oxidized flavin was used to estimate the FAD content of the protein. Table II shows that both activity and A_{450} absorption decrease as the ADPR concentration increases. In each case subsequent dialysis against an excess of free FAD restored the activity and the absorption at 450 nm completely. From these equilibrium dialysis experiments we derived for the equilibrium E·FAD + ADPR $\rightleftharpoons$ E·ADPR + FAD an equilibrium constant of about 1×10^{-3}. These results together with the interpretation of the difference Fourier maps indicate that an excess of ADPR replaces FAD from the enzyme.

Because ADPR can replace FAD from PHBH, we examined if FAD can also be replaced by other nucleotides that have an ADP moiety in common with FAD. The effect of various nucleotides on the binding of FAD in PHBH was determined with equilibrium dialysis. Table II shows that only ADPR exchanges FAD efficiently, while NAD and ADP exhibit a small effect on the FAD binding. The slow increase of the A_{280}/E_{450} ratio as a function of time, even after 48 h, is believed to be caused by slow denaturation of the enzyme because activities could not be fully restored by FAD after the experiment. Addition of the substrate did not influence the results significantly as is shown in Table III.

Course of Refinement and Final Model. After initial least-squares refinement without the terminal ribose of ADPR, this ribose was tentatively fitted to a blob of density extending from the pyrophosphate moiety. The model was further refined as is described under Materials and Methods. The position of the terminal ribose was not very stable during refinement, and its B values became high (around 50), indicating some disorder.

To get rid of as much model bias as possible, an $F_o - F_c'$ omit map was calculated in which the whole ADPR molecule was left out for the calculation of F_c'. This map is shown in Figure 2.

The electron density shows unambiguously the position of the ADP part of ADPR. From P2, density runs toward the terminal ADPR ribose moiety which is not very well resolved in the electron density. This, too, indicates some disorder at this ribose moiety. This is, however, not surprising since no real binding site is present for the ribose, so it might not have one single unique binding mode. The hydroxyl groups of the ribose moiety do not form optimal hydrogen bonds with protein ligands as is the case with the ribose moiety of the adenosine. In the final model (Figure 3), the ribose is sandwiched between the side chains of Asp 286 and Gln 102, two ligands of the ribityl chain in the native protein. The protein atoms and solvent molecules in the acitve-site region have a well-defined electron density in the final $2F_o - F_c$ map (Figure 3), except for the terminal ribose of the ADPR molecule.

Table IV gives the potential hydrogen-bond interactions between the ADPR terminal ribose, the enzyme, and the fixed

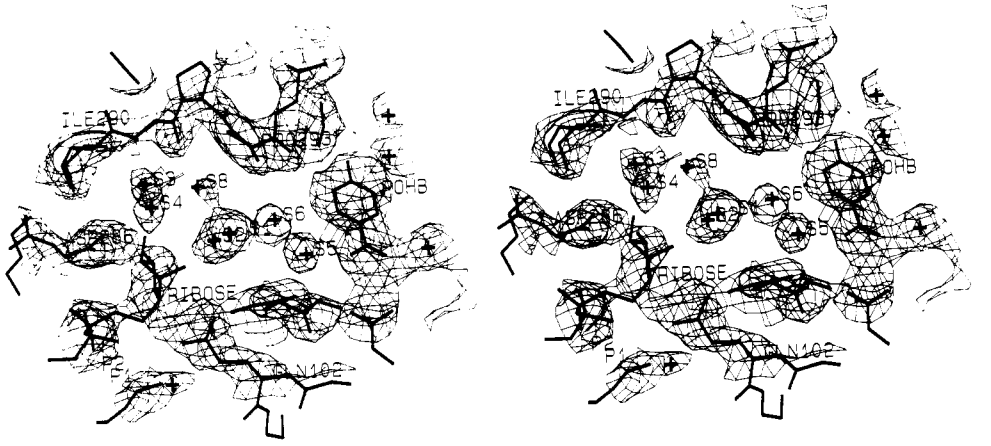

FIGURE 3: Stereoview of the binding pocket of the flavin ring in the σA weighted (Read, 1986) $(2F_o - F_c)$ exp $(i\alpha_c)$ map contoured at the 1σ level.

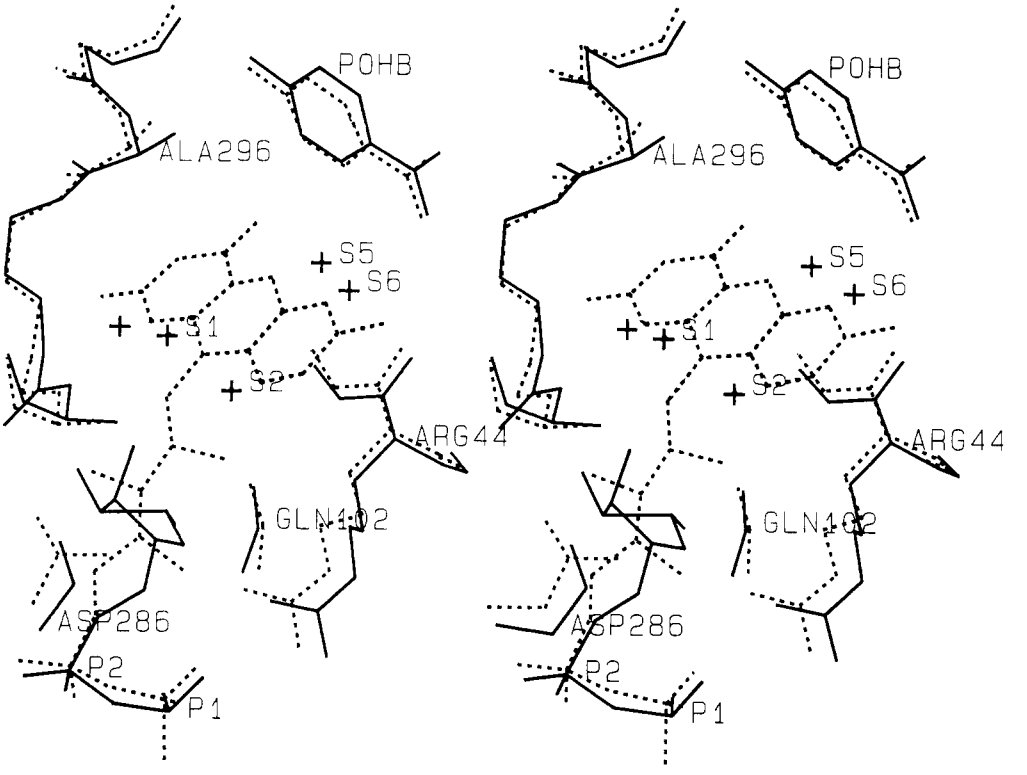

FIGURE 4: Stereoview of a superposition of the native PHBH·FAD·pOHB model (broken lines) onto the PHBH·ADPR·pOHB model (solid lines). Crosses denote the position of bound solvent atoms in the pseudo-apo model.

solvent molecules in the flavin binding pocket of the pseudo-apo-PHBH. Nine solvent molecules were located near the binding pocket of the flavin ring. Three of them (S3, S4, and S8) are also present in the structure of the native ES complex. The remaining six solvent molecules fill up the empty space, normally occupied by the flavin ring. Except for a rearrangement on the order of 1 Å of the side chain of Asp 286, no significant differences were found in the active site in the presence or absence of the flavin ring.

The electron density around solvent molecules S1 and S2 is rather high for a simple solvent like water, and in addition, the densities for S1 and S2 are always mutually connected. In the omit map (Figure 2) density smears out toward the ribose molecules. Solvent molecules S1 and S2 might therefore represent a more bulky molecule like sulfate, phosphate, or perhaps dithionite, which were present in high concentrations in the crystallization medium. S1 and S2 are bound at a site occupied in the native enzyme by the region of the flavin ring around N10. The proximity of the positive end of the dipole of helix H10 suggests that a negatively charged ion might occupy this site.

Discussion

Equilibrium dialysis experiments of PHBH against a solution of ADPR give rise to concomitant loss of activity and absorption at 450 nm. This can be restored by an excess of FAD. The crystal structure shows that in fact ADPR replaces the FAD cofactor from the enzyme, resulting in a pseudo-apoenzyme in which only the riboflavin moiety is absent.

From the native enzyme it is known that the flavin is bound rather tightly with a dissociation constant of 45 nM (Müller & Van Berkel, 1983). Therefore, in experiments aimed at the preparation of apoenzyme for reconstitution experiments, rather crude conditions such as high ionic strength, low pH, or the addition of denaturants had to be used to remove the FAD effectively. For instance, Entsch et al. (1980) recovered reconstitutable apo-PHBH from a precipitate of the enzyme in 3.0 M ammonium sulfate by lowering the pH to about 2.

Table IV: Potential Hydrogen-Bond Interactions between the ADPR Terminal Ribose, the Enzyme, and the Fixed Solvent Molecules in PHBH Complexed with ADPR and pOHB[a]

atom 1		atom 2	
ADPR terminal ribose			
O1	(3.0)	NE1	Arg 44
OH1	(2.7)	OD1	Asp 286
	(2.7)	S4	
	(3.3)	NE1	Arg 44
OH2	(2.6)	OD2	Asp 256
	(3.0)	S3	
OH3	(3.0)	S1	
	(3.5)	S2	
solvent			
S1	(2.8)	S2	
	(3.0)	OH3	terminal ribose
	(3.2)	N	Leu 299
	(3.2)	S8	
	(3.4)	S7	
S2	(2.8)	S1	
	(3.5)	OH3	terminal ribose
S3	(2.7)	N	Val 291
	(2.9)	S8	
	(3.0)	OH2	terminal ribose
	(3.1)	O	Val 291
	(3.2)	S4	
S4	(2.7)	OH1	terminal ribose
	(3.2)	S3	
S5	(3.0)	N	Gly 46
	(3.2)	O2*	pOHB
	(3.4)	S6	
S6	(3.4)	S5	
	(3.4)	S9	
S7	(2.8)	O	Val 47
	(3.4)	S1	
S8	(2.9)	O	Val 291
	(2.9)	S3	
	(3.2)	S1	
S9	(3.4)	S6	

[a] Distances in angstroms are given in parentheses. Solvent is abbreviated "S".

Müller and Van Berkel (1983) removed the FAD reversibly at neutral pH with a mixture of 2 M KBr and 2 M urea. In contrast to these rather extreme conditions, our equilibrium dialysis experiments show that ADPR is able to remove FAD from the enzyme under mild conditions and that the pseudo-apoenzyme formed can be reconstituted with FAD to regain fully active enzyme.

The initial protein solution that was used for the crystallization contained 0.16 mM PHBH with an A_{280}/A_{450} ratio below 10, together with 60 mM ADPR. Under the conditions of the dialysis experiments the amount of ADPR would be too low to remove the FAD completely. However, the crystallization conditions were rather extreme: 1.6 M ammonium sulfate, pH 4–6, and elevated temperature up to 37 °C for a rather long period of time.

The final electron density map shows that the ADP moiety of ADPR nicely fits the density. It superimposes very well with the original ADP moiety of FAD. The terminal ribose is less well resolved. This could indicate that its orientation in the ribityl binding pocket might be somewhat disordered because it does not fit tightly.

Because ADP itself can be fitted perfectly, the question remains why addition of ADP does not remove the FAD. It could be that in ADP the additional negative charge on the pyrophosphate, which is absent in ADPR, comes too close to the negatively charged side chain of Asp 286, which makes the interaction unfavorable in comparison with FAD and ADPR. When FAD is present, Asp 286 forms a hydrogen bond with one of the ribityl hydroxyl groups. With ADPR, Asp 286 is close to one of the terminal ribose hydroxyl groups. It is interesting to notice that in the crystal structure of trimethylamine dehydrogenase an ADP molecule was found inside a presumed FAD binding pocket (Mathews & Lim, 1987) and a solvent-filled cavity was present at the expected position of the riboflavin position of FAD. It is possible that replacement of FAD by nucleotide fragments can also occur in other FAD binding proteins.

Comparison of the structures PHBH·ADPR·pOHB and PHBH·FAD·pOHB around the flavin binding pocket, which is shown in Figure 4, shows that the protein structure is rather insensitive to the presence or absence of the flavin ring in the active site. The root mean square difference in coordinates for the Cα atoms between both complexes is 0.34 Å. This confirms the observations presented by Müller and Van Berkel (1983) which indicated that the binding sites for the substrate pOHB, the coenzyme NADPH, and the cofactor FAD must be very similar in native and apoenzyme. Unfortunately, the cocrystallization with ADPR did not give any clue for the possible NADPH binding site. Careful examination of the $(F_o - F_c)$ difference map after refinement did not reveal any additional ADPR molecule bound to the protein. So the binding mode of NADPH to PHBH remains an intriguing problem to be solved.

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Large Increases in General Stability for Subtilisin BPN′ through Incremental Changes in the Free Energy of Unfolding†

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ABSTRACT: Six individual amino acid substitutions at separate positions in the tertiary structure of subtilisin BPN′ (EC 3.4.21.14) were found to increase the stability of this enzyme, as judged by differential scanning calorimetry and decreased rates of thermal inactivation. These stabilizing changes, N218S, G169A, Y217K, M50F, Q206C, and N76D, were discovered through the use of five different investigative approaches: (1) random mutagenesis; (2) design of buried hydrophobic side groups; (3) design of electrostatic interactions at Ca^{2+} binding sites; (4) sequence homology consensus; and (5) serendipity. Individually, the six amino acid substitutions increase the ΔG of unfolding between 0.3 and 1.3 kcal/mol at 58.5 °C. The combination of these six individual stabilizing mutations together into one subtilisin BPN′ molecule was found to result in approximately independent and additive increases in the ΔG of unfolding to give a net increase of 3.8 kcal/mol (58.5 °C). Thermodynamic stability was also shown to be related to resistance to irreversible inactivation, which included elevated temperatures (65 °C) or extreme alkalinity (pH 12.0). Under these denaturing conditions, the rate of inactivation of the combination variant is ~300 times slower than that of the wild-type subtilisin BPN′. A comparison of the 1.8-Å-resolution crystal structures of mutant and wild-type enzymes revealed only independent and localized structural changes around the site of the amino acid side group substitutions. Consequently, the discovery that each independent stabilizing mutation makes a nearly additive contribution to the ΔG of unfolding has made it possible to dramatically increase the stability of subtilisin BPN′ in a coherent step by step manner, consistent with the model of "localization of free energy changes" for protein folding.

Factors that contribute to the stability of proteins have been enumerated and their relative importance understood in terms of the free energy difference between the native, N, and unfolded, U, states (Schellman, 1955; Kauzmann, 1959; Tanford, 1962, 1970; Brandts, 1964). The total free energy change for the reaction N ⇔ U has been described (Tanford, 1970) by the relation

$$\Delta G = \Delta G_{conf} + \sum_i \Delta g_{i,int} + \sum_i \Delta g_{i,s} + \Delta W_{el} \qquad (1)$$

where ΔG_{conf} = configurational free energy (order/disorder term), $\Delta g_{i,int}$ = short-range interactions (H-bonds, van der Waals interactions, salt bridges, cofactor binding, etc.), $\Delta g_{i,s}$ = short-range interactions with solvent (hydrophobic effect, hydration of ions, etc.), and ΔW_{el} = long-range electrostatic interactions (α-helix dipoles, etc.).

In this model for protein folding the symbol Δg_i represents

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