Stenkamp, R. E., Sieker, L. C., & Jensen, L. C. (1983) *J. Inorg. Biochem. 19*, 247–253.
Stenkamp, R. E., Sieker, L. C., & Jensen, L. H. (1984) *J. Am. Chem. Soc. 106*, 618–622.
Stenkamp, R. E., Sieker, L. C., Jensen, L. H., McCallum, J. D., & Sanders-Loehr, J. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 713–716.
Terwilliger, N. B., Terwilliger, R. C., & Schabtach, E. (1985) in *Blood Cells of Marine Invertebrates* (Cohen, W. D., Ed.) pp 193–225, Alan R. Liss, New York.
Thoai, N. V., & Robin, Y. (1969) in *Chemical Zoology* (Florkin, M., & Scheer, B. T., Eds.) Vol. IV, pp 163–203, Academic, New York.
Utecht, R. E. (1986) Ph.D. Thesis, Iowa State University.
Utecht, R. E., & Kurtz, D. M., Jr. (1985) *Inorg. Chem. 24*, 4458–4459.
Utecht, R. E., & Kurtz, D. M., Jr. (1988) *Biochim. Biophys. Acta 953*, 164–178.
Vasavada, K. V., Ray, B. D., & Rao, B. D. N. (1984) *J. Inorg. Biochem. 21*, 323–335.
Weber, R. E., & Fange, R. (1980) *Experientia 36*, 427–428.
Wilkins, R. G., & Harrington, P. C. (1983) *Adv. Inorg. Biochem. 5*, 51–85.
Wilkins, R. G., & Wilkins, P. C. (1987) *Coord. Chem. Rev. 79*, 195–214.

Crystallographic Analysis of the Binding of NADPH, NADPH Fragments, and NADPH Analogues to Glutathione Reductase

Emil F. Pai

Max-Planck-Institut für medizinische Forschung, Abteilung Biophysik, Jahnstrasse 29, D-6900 Heidelberg, Federal Republic of Germany

P. Andrew Karplus and Georg E. Schulz*

Institut für Organische Chemie und Biochemie der Universität, Albertstrasse 21, D-7800 Freiburg i.Br., Federal Republic of Germany

Received October 26, 1987; Revised Manuscript Received January 7, 1988

ABSTRACT: The binding of the substrate NADPH as well as a number of fragments and derivatives of NADPH to glutathione reductase from human erythrocytes has been investigated by using X-ray crystallography. Crystals of the enzyme were soaked with the compounds of interest, and then the diffraction intensities were collected out to a resolution of 3 Å. By use of phase information from the refined structure of the native enzyme in its oxidized state, electron density maps could be calculated. Difference Fourier electron density maps with coefficients $F_{soak} - F_{native}$ showed that the ligands tested bound either at the functional NADPH binding site or not at all. Electron density maps with coefficients $2F_{soak} - F_{native}$ were used to estimate occupancies for various parts of the bound ligands. This revealed that all ligands except NADPH and NADH, which were fully bound, showed differential binding between the adenine end and the nicotinamide end of the molecule: The adenine end always bound with a higher occupancy than the nicotinamide end. Models were built for the protein–ligand complexes and subjected to restrained refinement at 3-Å resolution. The mode of binding of NADPH, including the conformational changes of the protein, is described. NADH binding is clearly shown to involve a trapped inorganic phosphate at the position normally occupied by the 2′-phosphate of NADPH. A comparison of the binding of NADPH with the binding of the fragments and analogues provides a structural explanation for their relative binding affinities. In this respect, proper charge and hydrogen-bonding characteristics of buried parts of the ligand seem to be particularly important.

Glutathione reductase (EC 1.6.4.2) is a ubiquitous enzyme responsible for the maintenance of a high ratio of GSH/GSSG by the reaction GSSG + NADPH + $H^+ \rightleftharpoons$ 2GSH + $NADP^+$. In the course of the reaction the reduction equivalents are transferred from the nicotinamide to an FAD prosthetic group and then, via a redox-active disulfide bridge, to GSSG (Williams, 1976; Pai & Schulz, 1983). Glutathione reductase belongs to a family of structurally and mechanistically related enzymes that includes lipoamide dehydrogenase (Williams, 1976), asparagusate dehydrogenase (Yanagawa, 1979), trypanothione reductase (Shames et al., 1986; Krauth-Siegel et al., 1987), and mercuric reductase (Fox & Walsh, 1983). The kinetics of catalysis and the binding of nucleotides to the NAD(P)H binding site have been well studied for a number of these enzymes.

The structure of the oxidized, substrate-free form of glutathione reductase from human erythrocytes has recently been refined at 1.54-Å resolution (Karplus & Schulz, 1987), providing a context in which medium-resolution substrate binding studies can be interpreted with a reasonable level of accuracy. This paper describes the NADPH binding site as derived from 3-Å resolution studies of the enzyme complexed with either NADPH or other related ligands. The mode of binding to glutathione reductase is discussed in light of the results of

* To whom correspondence should be addressed.

0006-2960/88/0427-4465$01.50/0

kinetic studies and of the amino acid sequences of structurally related enzymes.

Materials and Methods

Glutathione reductase from human erythrocytes was purified and crystallized out of ammonium sulfate as described previously (Krohne-Ehrich et al., 1977). 2′P-5′ADP and 2′P-5′ADPme (see Table I for nomenclature) have been synthesized by Dr. R. Goody, Heidelberg. NADPH and the remaining fragments and derivatives of NADPH used in this study were purchased from Sigma Chemical Co. (St. Louis, MO). The conditions for the various soaking experiments are described in Tables I and II. The X-ray data collection, using a four-circle diffractometer (Modified $P2_1$, Nicolet Inc.), and the data reduction followed the protocol described by Thieme et al. (1981). For soaks A–N (Table I), complete 3-Å data sets divided into seven shells of equal volume in reciprocal space were collected from a single crystal. Data collection was terminated after a single shell at 5.8 Å for soaks P–W (Table II). For soaks yielding reduced glutathione reductase, EH_2 (see Table I for definition), the soak solutions were bubbled with nitrogen but were not strictly anaerobic. The capillary was filled with the soak solution during data collection, and the crystal was held in place with pipe-cleaner fibers. The quality of all data was comparable to that of native glutathione reductase (Thieme et al., 1981) with R factors between symmetry-related structure factors ranging from 3% to 10%.

For interpretation of the soaks, electron density maps were calculated with coefficients $F_{soak} - F_{native}$ and $2F_{soak} - F_{native}$ by using the phases from the 1.54-Å refinement of oxidized glutathione reductase (Karplus & Schulz, 1987). The $F_{soak} - F_{native}$ maps were used for qualitative analysis and for the location of unambiguous movements of protein atoms. In many cases this qualitative analysis clearly indicated that different parts of a single ligand showed different levels of binding. For instance, the difference map for $NADP^+$ (soak B, Table I) showed good density for the adenine base but almost no density for the nicotinamide.

We have quantified this phenomenon for various parts of the bound ligands using the following formula: Occupancy $= (\rho_{soak} - \rho_{control})/(\rho_{NADPH} - \rho_{control})$, where ρ is the electron density at a given atom position of the F_{native} map ($\rho_{control}$), of the $2F_{soakA} - F_{native}$ map (ρ_{NADPH}; soak A was with NADPH, see Table I), and of any of the $2F_{soak} - F_{native}$ maps (ρ_{soak}). This method approximates the density integration over whole groups by sampling at single points. The committed error is small under the assumption that the mobilities of all relevant groups are similar. For the phosphoryl groups we used the phosphorus positions, which have peak electron densities at 3-Å resolution. The occupancy of the nicotinamide moiety could only be determined indirectly, because on binding, nicotinamide replaces the side chain of Tyr-197 that moves to a region previously filled with bulk solvent. We therefore took the density increase at the new position of Tyr-197 (Tyr-197-out) as a measure for nicotinamide binding. In particular, we used the average of the densities at the CE1, CE2, CZ, and OH atom positions of Tyr-197-out for deriving the ρ values of the formula above.

A comparison showed that the average density at Tyr-197-out in the $2F_{soakA} - F_{native}$ map ($=\rho_{NADP}$) was approximately equal to the corresponding densities of four arbitrary ordered tyrosines (Tyr-21, Tyr-23, Tyr-106, Tyr-114) in the F_{native} map. This suggests that for soak A, Tyr-197-out and thus nicotinamide are fully occupied. Accordingly, we conclude that the relative occupancies of Table I correspond closely to absolute occupancies. This conclusion is supported by the observation that no other soak shows significantly higher occupancies (Table I). The accuracy of the method has been estimated as 5–10 occupancy percentage points on the basis of scatter of the densities observed for selected protein atoms, which were unaffected by ligand binding, in the various $2F_{soak} - F_{native}$ maps.

Model building was done with reference to the $2F_{soak} - F_{native}$ maps by using an interactive computer graphics system described earlier (Karplus & Schulz, 1987). The bound ligand and the new positions of Tyr-197, Ile-198, and Arg-218 were introduced into the protein model established with the 1.54-Å refinement. All atoms of these groups were assigned temperature factors of 20 $Å^2$. The new protein models together with all atoms of the ligand with occupancies greater than 35% were subjected to restrained crystallographic refinement using the TNT program package of Tronrud et al. (1987). The coordinates were refined against the X-ray data from 10- to 3-Å resolution by using the final weights and, except as noted above, the temperature factors of the 1.54-Å refinement (Karplus & Schulz, 1987). Ideal geometry for nicotinamide was taken from Wright and King (1954). For each soak, the refinement was judged to be complete after about 10 cycles. The final R factors were always between 12% and 14%. As a control, the coordinates of the original unliganded model were refined against the native data truncated at 3-Å resolution. This refinement suggested that for the analysis of the liganded structures any movement greater than 0.3 Å can be considered significant.

Results and Discussion

(A) Binding of NADPH. Two independent soaks with NADPH were made (soaks A and A′, Table I). in the first, full occupancy was seen, while the second showed a lower occupancy for the nicotinamide half of the molecule. It is probable that soak A′ had too much oxygen present and that $NADP^+$ produced by the oxidase activity of glutathione reductase displaced some of the NADPH (see discussion of $NADP^+$ binding below). Despite the high concentration of NADPH used in these soaks, no evidence of secondary binding sites was seen. This observation also holds for the other compounds used in this study.

The difference electron density for NADPH as bound to glutathione reductase is shown in Figure 1a. The density for the nicotinamide is not very clear because Tyr-197 occupies this position in the enzyme without substrate. The $2F_{soak} - F_{native}$ synthesis shown in Figure 1b overcomes the problems caused by displaced protein or solvent atoms (Blundell & Johnson, 1976) to provide the true electron density for the bound substrate. It can be easily interpreted and identifies the functional NADPH binding site on the enzyme. The difference map also had large peaks in the region of the redox-active disulfide bridge, due to the reduction of this disulfide by NADPH. However, a comparison with difference maps of the substrate-free reduced enzyme (EH_2) showed that these changes are independent of substrate binding. They will not be discussed in this paper.

NADPH is bound in an extended conformation in a pocket on the surface of the enzyme (Figure 2). Most of its contacts are with residues of the NADP domain (Thieme et al., 1981), but the nicotinamide extends forward to stack on the flavin ring system. At this position, residues from the FAD domain, the central domain, and the interface domain come together to participate in binding. As is commonly found for dinucleotides, the pyrophosphate bridge is bound at the C-terminal end of a parallel β-sheet (Rossmann et al., 1975). The α-helix beginning at residue 196 is positioned to stabilize the negative charge of the pyrophosphates (Hol et al., 1978). The

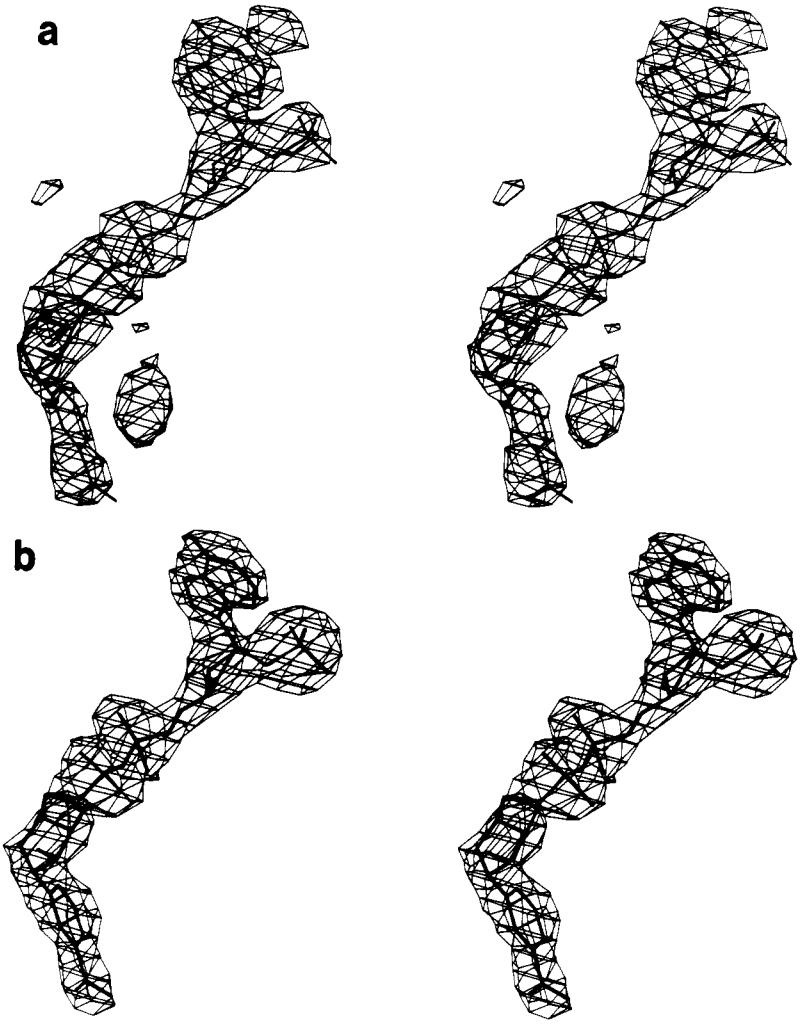

FIGURE 1: Electron density at 3-Å resolution and the refined model of NADPH bound to glutathione reductase. (a) Positive difference electron density ($F_{NADPH} - F_{native}$) exp($i\alpha_{native}$) contoured at 25% of the maximal electron density (ca. 5 rms). The density signal for the nicotinamide is not complete because Tyr-197 was bound at that position. Additional major peaks of density next to the nicotinamide and adenine are due to the new positions of Tyr-197 and Arg-218, respectively. Also seen is density for two new solvent molecules near the N-phosphate. (b) Electron density from the ($2F_{NADPH} - F_{native}$) exp($i\alpha_{native}$) map contoured at 14% of the maximum. For clarity, all density further than 2 Å away from NADPH atoms has been removed. This map was used for the initial fitting and gives accurate density for the nicotinamide ring. Note also how the 2′-phosphate density is much stronger than in the difference Fourier map above. This is due to the presence of the inorganic phosphate at this position in the unliganded enzyme.

FIGURE 2: CA backbone of a subunit of glutathione reductase with bound FAD and NADPH. Residues 18 (the visible amino terminus) and 195 (in the NADPH binding domain) are labeled.

topology of the NADP domain and its similarity to the FAD domain of glutathione reductase and to the FAD domain of *p*-hydroxybenzoate hydroxylase have been discussed in detail by Wierenga et al. (1983).

The torsion angles of bound NADPH are given in Table III together with the corresponding values for NADPH on dihydrofolate reductase and for $NADP^+$ bound to catalase. Experience indicates that the torsion angles of NADPH de-

Table I: Summary of Ligand Binding Experiments at 3-Å Resolution

soak	ligand[a]	concn (mM)	K^b (μM)	time[c] (days)	enzyme form[d]	approximate occupancies[e] (%) PR	PA	PN	Tyr-197 at "out" position
A	NADPH	10	8	0.02	EH_2	100	100	100	100
A′	NADPH	6	8	0.02	EH_2	103	96	89	77
B	$NADP^+$	10	70	17	E	76	64	20	15
	GSSG	10	65						
C	$NADP^+$	10	70	4	EHR	74	58	32	17
D	2′AMP	10	500	19	E	44	(−13)	(1)	(5)
E	2′P-5′AMP	10	30	2	E	100	77	(2)	(0)
F	2′P-5′ADP	10	5000	5	E	27	13	3	(3)
G	2′P-5′ADPme	20	150	18	E	36	40	36	(−3)
H	2′P-5′ADPrib	10	75	0.8	E	90	66	53	29
I	2′P-5′ADPrib	5[f]	75	6	EH_2	86	68	40	16
J	NADH	10	500	0.7	EH_2	100[g]	100	94	97
K	$amNADP^+$	5[f]	50	12	EH_2	90	75	57	51
L	thNADP(H)	5[f]	200	0.9	EH_2	77	66	49	36
M	acNADP(H)	5[f]	400	18	EH_2	50	34	17	10
N	etNADP(H)	2.5[f]	2000	49	EH_2	33	15	14	10

[a] Nonstandard abbreviations: 2′AMP, adenosine 2′-monophosphate; 2′P-5′AMP, adenosine 2′-monophosphate 5′-monophosphate; 2′P-5′ADP, adenosine 2′-monophosphate 5′-diphosphate; 2′P-5′ADPme, adenosine 2′-monophosphate 5′-diphosphate β-monomethyl ester; 2′P-5′ADPrib, adenosine 2′-monophosphate 5′-diphosphate β-5′-ribosyl ester; $amNADP^+$, 3-aminopyridine adenine dinucleotide phosphate; thNADP(H), thionicotinamide adenine dinucleotide phosphate; acNADP(H), 3-acetylpyridine adenine dinucleotide phosphate; etNADP(H), nicotinamide N^{10},N^1-ethenoadenine dinucleotide phosphate. [b] Either K_m for NADPH, NADH, and GSSG or K_i is given. The values for NADPH, $NADP^+$, and GSSG are from Worthington and Rosemeyer (1976). The remaining values are from Pai et al. (1984). [c] All soaks were carried out in 2 M $(NH_4)_2SO_4$, 0.1 M potassium phosphate, pH 7.0 at 4 °C. Data collection took ca. 7 and 1 days for 3- and 5.8-Å resolution analyses, respectively. [d] E is the oxidized enzyme, EH_2 is the two-electron-reduced enzyme in which the redox-active disulfide is reduced, and EHR (Arscott et al., 1981) is an inactive enzyme form locked into the reduced state by carboxamidomethylation of Cys-58. EHR was prepared by reducing crystals for 30 min in 2 mM dithioerythritol (DTE) at 4 °C and then allowing them to react 150 min at room temperature with a solution of 1 mM DTE and 2 mM iodoacetamide. The reaction was stopped by transferring the crystals to 100 mM DTE for 15 min and then to 50 mM DTE for an additional 15 min. The crystals were then washed 3 times for 15 min each in artificial mother liquor and stored in the same until use. The modification was stable for more than a year. Both EH_2 and EHR have a reddish brown rather than yellow color. [e] The method for calculating these occupancy values is given under Materials and Methods. Entries are enclosed in parentheses if the given ligand is missing the corresponding part of NADPH such that the values should be zero. [f] These soaks also included 0.5–1.0% β-mercaptoethanol. [g] This corresponds to the inorganic phosphate bound slightly displaced from the 2′-phosphate position.

Table II: Summary of Ligand Binding Experiments at 5.8-Å Resolution

soak	ligand[a]	concn (mM)	K^b (mM)	time[c] (days)	enzyme form[d]	comment on visual inspection of binding[e]
P	NAD^+	10	2.9	1.5	E	density at adenine site
Q	NAD^+	10	2.9	4	EHR	density at adenine site
R	NAD^+	10[f]	2.9	0.7	EH_2	proper (about 30% of soak S)
S	NADH[g]	10[f]	0.5	0.7	EH_2	proper
T	5′AMP	10	12	35	E	density at adenine site
U	5′ADPrib	20		4	E	density at adenine site
V	5′ADPrib	10[h]		2	E	density at adenine site
	nicotinamide	15				
W	nicotinamide	100		0.8	E	density at adenine site

[a] Nonstandard abbreviation: 5′ADPrib, adenosine 5′-diphosphate β-5′-ribosyl ester. [b,c,d] As in Table I. [e] Based on inspection of the $F_{soak} - F_{native}$ difference electron density maps. [f] The soak also included 0.5–1.0% β-mercaptoethanol. [g] Data from soak J (Table I) analyzed with a 5.8-Å resolution cutoff. [h] The experiment started after soaking for 1 day in 100 mM nicotinamide.

termined here with reference to 3-Å resolution data have a significant level of error (Filman et al., 1982; Karplus & Schulz, 1987). However, even when this is taken into account, it seems that NADPH on glutathione reductase takes on a conformation significantly different from those of the other two known cases. In glutathione reductase, the adenine and nicotinamide rings are both in the anti conformation. The nicotinamide assumes the more common position nearly trans to the C1′N–O4′N bond, while the adenine is nearly trans to C1′A–C2′A. The identification of the adenine ribose pucker as 3′-endo is quite reliable because the presence of the 2′-phosphate group aids interpretation. The conformation of the nicotinamide ribose is not quite so certain, but both the electron density distribution and the geometry of possible hydrogen-bonded contacts indicate a 3′-endo structure. The conformation through the pyrophosphate bridge is rather extended. Despite the fact that only one of the relevant torsion angles, θ_N, is near the trans position, the C4′A to C4′N distance, at 8.8 Å, is 85% of the maximum possible distance.

In the following section, the interactions of each part of NADPH with the enzyme will be presented, starting with the adenine group and ending with the nicotinamide group. The nomenclature used is given in Table III. Figure 3 shows a stereo view of the protein environment of bound NADPH, and Table IV lists the major enzyme–ligand contacts. This section also includes a discussion of the substitution pattern of the involved amino acid residues in the sequences of related NAD(P)H binding enzymes (see Table IV).

Adenine (A). As seen in Figure 3, the adenine moiety is bound sandwiched between the side chains of Ile-289 and Arg-218. Arg-218 is quite mobile in the structure of substrate-free enzyme and interacts loosely with Gln-250. Upon binding of NADPH, this interaction is broken, and the guanidinium group relocates to bind to the 2′-phosphate group. In this new position the planar guanidinium group assumes a position approximately parallel to and 3.5 Å away from the adenine. Since the substrate-free enzyme already had a phosphate bound at the position of the 2′-phosphate (Karplus

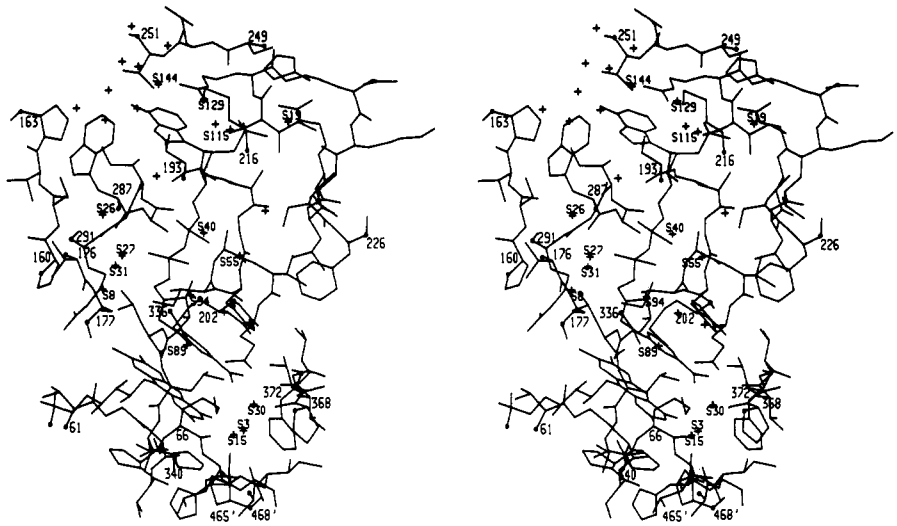

FIGURE 3: Stereo view of the complete NADPH binding site. Chain segments, with sequence in parentheses, included are 61–68 (VGCVPKKV), 160–163 (PSTP), 176–177 (TS), 193–202 (VGAGYIAVEM), 216–226 (MIRHDKVLRSF), 249–251 (SQV), 287–291 (WAIGR), 336–340 (ALLTP), 368–372 (PTVVF), and 465′–468′ (AIHP). The points at which the chains are cut are marked with dots. Also included are bound solvent sites modeled as oxygen atoms (+). The most tightly bound solvent molecules are labeled with their identifying numbers (S3 = Sol-3, S8 = Sol-8, etc.).

Table III: Conformation of Enzyme-Bound NADP

torsion angle name	angle definition[a]	GR[b] (deg)	DHFR[c] (deg)	CAT[d] (deg)
χ_A	C4A-N9A-C1′A-C2′A	147	105	139
	A-ribose pucker[e]	^{3}E	^{3}E	^{2}E
ξ_A	C3′A-C4′A-C5′A-O5′A	−33	−169	−25
θ_A	C4′A-C5′A-O5′A-PA	128	−163	−173
θ'_A	C1′A-C2′A-O2′A-PR	89	145	125
ψ_A	C5′A-O5′A-PA-OAN	88	−72	37
ϕ_A	O5′A-PA-OAN-PN	−93	141	144
ϕ_N	O5′N-PN-OAN-PA	−117	82	−161
ψ_N	C5′N-O5′N-PN-OAN	103	57	−109
θ_N	C4′N-C5′N-O5′N-PN	−153	129	118
ξ_N	C3′N-C4′N-C5′N-O5′N	19	−172	−54
	N-ribose pucker[e]	^{3}E	^{2}E	^{2}E
χ_N	C2N-N1N-C1′N-C2′N	72	117	−54

[a] Individual atoms (e.g., C4A and C2N) or groups of atoms (e.g., A-ribose and N-ribose) are designated A or N depending on their belonging to the adenine or nicotinamide part of the dinucleotide, respectively. A terminal R designates atoms belonging to the ribosyl- or 2′-phosphate. F is used for flavin. [b] NADPH bound to human erythrocyte glutathione reductase (this work). [c] NADPH bound to dihydrofolate reductase (Filman et al., 1982). [d] $NADP^+$ bound to catalase (Fita & Rossmann, 1985). [e] Only 2′-endo (^{2}E) and 3′-endo (^{3}E) conformations are considered.

& Schulz, 1987), it seems that the observed stacking interaction with the adenine ring system plays an important role in stabilizing the new position of Arg-218. A similar favorable stacking interaction of Arg-224 with His-219 is seen at the 2′-phosphate binding site (see Figure 3).

Arg-218 is conserved in all of the closely related NADPH binding enzymes but not in lipoamide dehydrogenase, which binds NADH. Here, it is replaced by a methionine that could also provide a hydrophobic surface for adenine. On the other side of the adenine sits Ile-289, for which the torsion angle χ_2 is near 180°, such that it produces a relatively flat surface for van der Waals contact. In mercuric reductase, the equivalent residue is threonine (Table IV). Sequence comparison studies would not consider Ile ↔ Thr a conservative substitution, but in this case it is structurally conservative as the polar hydroxyl of a threonine would point away from the adenine (Figure 3). For NADH bound by horse liver alcohol dehydrogenase, both sides of the adenine are covered by isoleucines (Eklund et al., 1984). In dihydrofolate reductase, His-64 and Leu-62 sandwich the adenine in a similar way (Filman et al., 1982).

There are no hydrogen bonds between protein atoms and the adenine. Rather, the edges of the adenine are quite exposed and surrounded by a number of loosely bound water molecules. Low-resolution studies with NADPH fragments that are very poor inhibitors of the enzyme all show binding only in the adenine pocket (Table II). The nonspecific nature of the binding is indicated by the binding of nicotinamide in soak W (Table II).

A-Ribose and 2′-Phosphate. That the 2′-phosphate makes a large contribution to the binding of NADPH is indicated by the about 60-fold weaker binding of NADH to glutathione reductase. The binding site of the 2′-phosphate is made up by the side chains of Arg-218, His-219, and Arg-224, along with three tightly bound solvent molecules (Table IV). Arg-218 and Arg-224 make multiple short contacts, while His-219 is somewhat farther removed. This environment suggests that the bound phosphate is dianionic, as has been shown for dihydrofolate reductase (Feeney et al., 1975).

In the related enzymes, Arg-218 and Arg-224 are well conserved, while His-219 is a lysine in glutathione reductase from *Escherichia coli* and an asparagine or a serine in the known mercuric reductase sequences. As expected, none of these residues are conserved in lipoamide dehydrogenase. No helices are involved in stabilizing this phosphate group. The 3′-hydroxyl group of the ribose points away from the enzyme and is surrounded by bulk solvent. O4′A however, is buried and receives an H-bond from 195-N.

Pyrophosphate. The pyrophosphate group is bound such that the N-phosphate is buried and the A-phosphate is exposed to bulk solvent (Figure 3). Table IV shows that the N-phosphate interacts with five different residues, while the A-phosphate only makes contact with one residue, Gly-196. The approach of Gly-196 to the A-phosphate is so close that a CB atom could not be accommodated. Surprisingly, a serine occupies this position in mercuric reductase, which probably causes its main chain to adopt a slightly different conformation in this region. The only direct H-bond between pyrophosphate and polypeptide is between an oxygen of the N-phosphate and 198-N. As discussed by Wierenga et al. (1985), such a hydrogen bond with the third residue of the pyrophosphate-binding α-helix is a common feature of the nucleotide binding

Table IV: Interactions between NADP and Human Glutathione Reductase[a]

NADP group[b]	protein group	probable H-bond	residues in the sequences of[c] GReco	MR pI258	MR tn501	LipDH
adenine	Sol-115	+				
	Arg-218 (side)		+	+	+	Met
	Ile-289 (all)		+	Thr	Thr	+
	Val-193 (side)		+	Ile	Ile	Met
	Gly-194 (main)		+	+	+	+
A-ribose	Ala-195 (all)		+	Ser	Ser	Gly
	Gly-196 (main)		+	+	Ser	+
	Ala-288 (main)		+	+	+	+
	Gly-194 (main)		+	+	+	+
	Arg-218 (side)		+	+	+	Met
	Ile-289 (main)		+	Thr	Thr	+
2′-phosphate	Sol-115	+				
	Sol-19	+				
	Sol-90	+				
	Arg-224 (side)	+	+	Lys	+	Pro
	Arg-218 (side)	+	+	+	+	Met
	His-219 (side)	+	Lys	Ser	Asn[d]	Phe
	Ala-195 (side)		+	Ser	Ser	Gly
A-phosphate	Sol-55	+				
	Gly-196 (main)		+	+	Ser	+
N-phosphate	Sol-27	+				
	Ile-198 (all)	+	+	+	Val	+
	Sol-40	+				
	Gly-290 (main)		+	+	+	+
	Tyr-197 (all)		+	+	Val	Ile
	Gly-196 (main)		+	+	Ser	+
	Arg-291 (side)		+	+	+	+
N-ribose	Leu-337 (all)	+	+	Gln	Gln	Met
	Sol-14	+				
	Sol-94	+				
	Arg-291 (side)		+	+	+	+
	Tyr-197 (side)		+	+	Val	Ile
	Ile-198 (side)		+	+	Val	+
	FAD					
nicotinamide	Glu-201 (side)	+	+	+	+	+
	Val-370 (main)	+	+	+	+	Ile
	Sol-30	+				
	Ile-198 (side)		+	+	Val	+
	FAD					
	Tyr-197 (side)		+	+	Val	Ile
	Lys-66 (side)		+	Ser	Ser	Ser
	Phe-372 (side)		+	+	+	Tyr
	Leu-338 (side)		+	Phe	Phe	+

[a] Amino acid residues and tightly bound solvent molecules are listed that are within 4 Å of a non-hydrogen atom of NADPH. All solvent atoms have been modeled as water and are virtually unmoved from their positions observed in the unliganded structure (Karplus & Schulz, 1987). For amino acid residues a distinction is made between side-chain and main-chain involvement; "all" denotes main and side. [b] See Table III for nomenclature. [c] Enzymes using NADP are GReco, glutathione reductase from *E. coli* (Greer & Perham, 1986) and MR, mercuric ion reductase coded by *Staphylococcus aureus* plasmid pI258 (Laddaga et al., 1987) in the first column and tn501 (Brown et al., 1983) or tn21 (Misra et al., 1985) in the second column. The enzyme LipDH, lipoamide dehydrogenase from *E. coli* (Stephens et al., 1983), uses NAD. A plus sign indicates that the sequence is the same as in glutathione reductase from human erythrocytes (Krauth-Siegel et al., 1982). [d] At this position tn21 has Ser.

fold. There are no positively charged side chains near the pyrophosphate.

N-*Ribose*. The furanose oxygen of this ribose is buried, and the 2′- and 3′-hydroxyl groups stick out toward the solvent. O3′N may make an intramolecular H-bond to O1A of the pyrophosphate group, helping to fix the relative orientation of the ribose group. Of the remaining three H-bonds formed by the ribose atoms, two involve tightly bound solvent molecules that are buried upon NADPH binding. The remaining H-bond is from O2′N to 337-O.

Nicotinamide (N). The nicotinamide group itself sits snugly in the pocket vacated by the side chain of Tyr-197 (Figure 3). It is nearly parallel to and approximately 3.5 Å away from the middle ring of the flavin. The rings have nearly the same orientation so that N1N is above N10F and C4N is above N5F. The stereochemistry is such that the H_s-atom of the nicotinamide can be transferred to the *re* face of the flavin (Pai & Schulz, 1983). This is in accordance with isotopic labeling studies (Stern & Vennesland, 1960; Manstein et al., 1986).

The orientation of the carboxamide group can be deduced from its environment. N7N is positioned to donate H-bonds to the rigidly held protein atoms 370-O and Glu-201-OE1, and O7N accepts a single H-bond from Sol-30. The carboxamide group is also stacked on the flavin with O7N sitting above N3F and N7N above O4αF. The carboxamide group is slightly twisted relative to the pyridine ring (ca. 10°) such that O7N is somewhat closer to the flavin than is N7N. The backside of the nicotinamide ring is covered by the repositioned side chain of Tyr-197 and is mostly shielded from solvent.

Most of the pyridine ring is in a rather nonpolar environment. However, C4N, which gives up a hydride ion during oxidation, is positioned around 3 Å from the salt bridge between Lys-66-NZ and Glu-201-OE1. The close proximity of C4N to 66-NZ could explain the lower affinity of the enzyme for oxidized versus reduced NADP. All of the residues involved in nonpolar contacts with the nicotinamide are either conserved or replaced by other nonpolar residues in related enzymes (Table IV). Glu-201 is strictly conserved, while Lys-66 is replaced by a serine in both mercuric reductase and lipoamide dehydrogenase. Interestingly enough, a serine at this position could possibly make a hydrogen bond to 201-OE2 with only minor changes in the main chain, as the distance from 66-CG to 201-OE2 is only 3.5 Å. The idea that the conserved Lys-67 could curl back to replace Lys-66 (Rice et al., 1984) seems less likely, because Lys-67 cannot reach the same position as Lys-66. Moreover, Lys-67-NZ is involved in an important intersubunit hydrogen bond to *cis*-Pro-468′-O.

(*B*) *Induced Fit*. It has long been recognized that an enzyme need not be a rigid molecule that has a perfectly preformed substrate binding site but that the interaction with substrate may induce a structural change in the enzyme which optimizes the binding of substrate (Koshland, 1958). In this study, a movement of greater than 0.3 Å can be considered significant (see Materials and Methods), and only atoms fairly directly involved in NADPH binding exceed this limit. Only two side chains undergo major conformational changes (see Figure 1a). For Arg-218 the side-chain torsion angle χ_3 changes from near 60° to near 300°, such that the NH2-atom moves by 5.6 Å from near the end of Gln-250 toward the 2′-phosphate group. Despite the contact made with Gln-250, the old position of Arg-218 was not particularly stable. It was very exposed to solvent and relatively mobile with temperature factors around 50 Å^2. At its new position, however, it is covered by the adenine group of NADPH (see above) and has quite well defined density.

The other side chain that undergoes a major change in conformation is Tyr-197, which occupies the nicotinamide binding pocket in the unliganded enzyme. The changes undergone in this region of the structure are shown in Figure 4. For Tyr-197 there is main-chain motion plus a rotation around the CA–CB bond from $\chi_1 = 90°$ to $\chi_1 = 280°$. The terminal atom, 197-OH, moves by 6.4 Å. In contrast to the situation for Arg-218, both the original and the new positions assumed

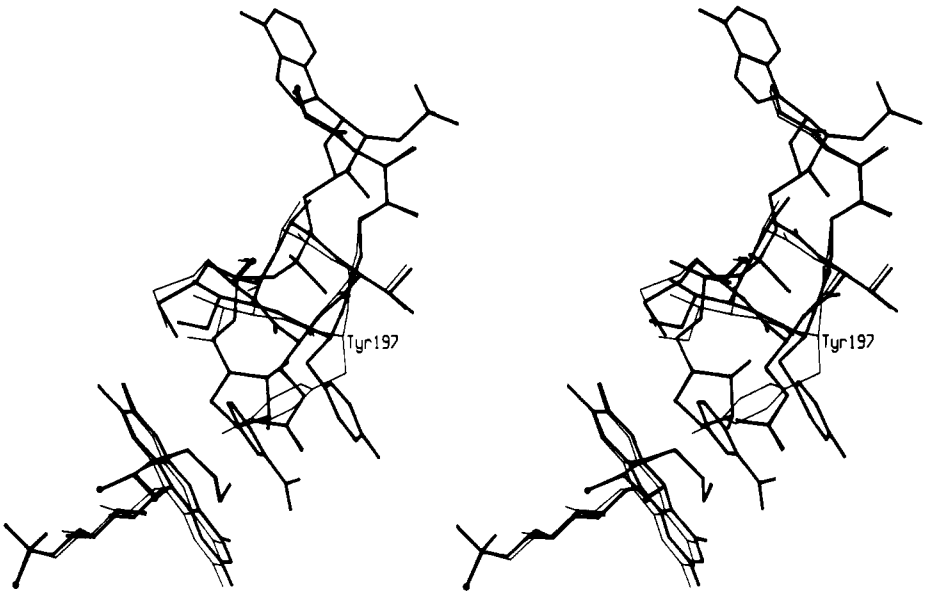

FIGURE 4: Stereo view of the bound NADPH molecule and the residues that are involved in the induced fit at the nicotinamide binding pocket. The thin lines are the unliganded structure (Karplus & Schulz, 1987), and the thick lines represent the liganded conformation. Shown are Lys-66 (behind the flavin), residues 194–202 (sequence GAGYIAVEM), the FMN moiety of FAD, and NADPH. Tyr-197-CA is labeled, and places where the chain has been cut are marked by dots.

by Tyr-197 are relatively well ordered. Possible strain in the original position is indicated by the χ_1 angle of 90°, which is 30° away from the staggered conformation $\chi_1 = 60°$. Also as noted by Karplus and Schulz (1987) the conformation around $\chi_1 = 60°$ is unfavorable for large side chains. It places the side chain gauche to both peptide N and C atoms. Among 12 tyrosine residues in glutathione reductase, Tyr-197 is the only one that has this conformation. It seems that the movement of this tyrosine to an energetically more favorable conformation would contribute to the strength of NADPH binding.

Accompanying the movement of Tyr-197 is a small rotation of the α-helical residues 196–201 (Figure 4). In this way, the side chain of Ile-198 moves as a rigid group about 0.5 Å away from the incoming nicotinamide to avoid close contacts. A smaller but still significant movement upon NADPH binding occurs for the main chain near Gly-290, which moves about 0.5 Å away from the pyrophosphate group of NADPH. Further details of this change in conformation will have to await analysis at higher resolution.

The sequence of events involved in the two large conformational changes cannot be derived from this analysis of the two static structures. For Arg-218, there is some disorder in the unliganded state, indicating that some molecules assume the liganded conformation. This suggests that NADPH may not be directly necessary for the change but may select enzyme molecules with the preexisting favorable conformation. For Tyr-197, such disorder is not observed, and the conformational change is probably specifically triggered during the binding of substrate. Studies with fragments of NADPH, which are described below, address this point further.

(*C*) *Binding of NADP*$^+$. The binding of $NADP^+$ to the oxidized form of the enzyme was examined in soak B (Table I). The 2′P-5′AMP end of $NADP^+$ binds in exactly the same way as has already been described for NADPH. However, the remaining part of the molecule is rather disordered. On the basis of a quantitative analysis of the electron density, the 2′P-5′AMP end of the molecule bound with an occupancy of higher than 70%, while the NMP half of the cofactor bound properly to about 15% of the enzyme molecules (Table I). Although the side chain of Tyr-197 occupies, on average, its new position away from the nicotinamide pocket 15% of the time, it is impossible to tell to what extent the nicotinamide pocket is filled by the nicotinamide instead of solvent molecules. Weak electron density adjacent to the A-phosphate indicates an alternate position of the N-phosphate, pointing away from the enzyme surface into the solvent region. It is apparently unfavorable for the positively charged oxidized nicotinamide ring to be buried in the nicotinamide pocket. The most likely reason for this is an unfavorable electrostatic interaction with the ammonium group of Lys-66.

In soak C, the binding of $NADP^+$ to the reduced form of the enzyme was investigated. In this experiment carboxamidomethylated enzyme (EHR) was used instead of the reduced enzyme (EH_2) to ensure that the $NADP^+$ could not be converted to NADPH by the reverse reaction. When the difference electron density is contoured at a relatively low level, the movement of Tyr-197 can be seen, leading to the conclusion that $NADP^+$ binds properly to the reduced enzyme (Pai & Schulz, 1983). However, the $2F_{soak} - F_{native}$ density shows that the binding of the nicotinamide is similar to that observed for the oxidized enzyme (Table I). Therefore, one has to conclude that although EH_2 and EHR have an extra negative charge from the thiolate at the flavin (Williams, 1976), it does not suffice to compensate the positive charge of the nicotinamide of $NADP^+$ at the other face of the flavin.

The characteristics of the binding of $NADP^+$ may provide a simple physical explanation for the results of kinetic studies (Carlberg & Mannervik, 1986) which show that $NADP^+$ acts to stimulate the rate of electron transfer to 2,4,6-trinitrobenzenesulfonate (TNBS). Soak C demonstrates that $NADP^+$ causes Tyr-197, which may act as a protective lid covering the flavin (Schulz et al., 1982), to move away, even though the nicotinamide does not necessarily take its place. This would leave the nicotinamide pocket empty and the *re* face of the flavin open for electron acceptors like TNBS. In this way, $NADP^+$ would act as a positive effector for TNBS reduction. In light of this hypothesis it would be interesting to test the effect of 2′P-5′ADPrib on TNBS reduction. As this compound displaces Tyr-197 (see soaks H and I of Table I) and cannot fill the nicotinamide pocket, it should be even more capable than $NADP^+$ of exposing the flavin.

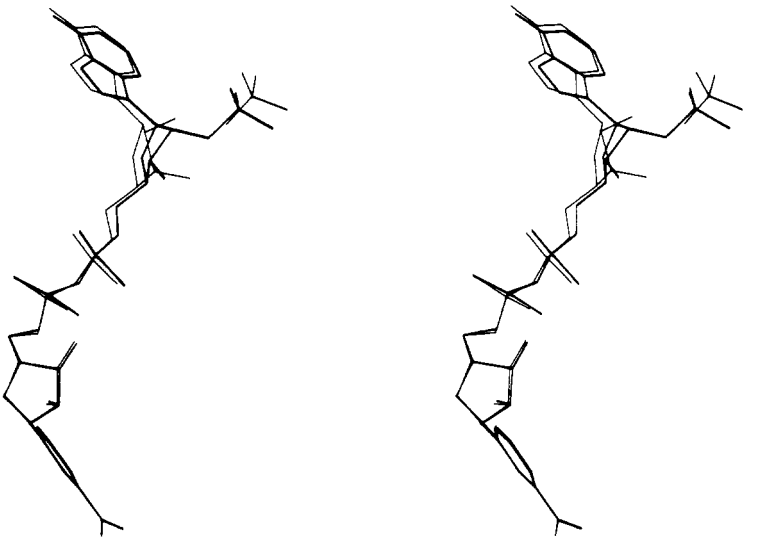

FIGURE 5: Positions of bound NADH and inorganic phosphate (thin lines) as compared to NADPH (thick lines).

(*D*) *Binding of NADH.* NADH is also a substrate for glutathione reductase but has a K_m about 60 times higher than that of NADPH. Despite this, the concentration used for soaking the crystal was high enough to provide full saturation of the binding site (see soak J, Table I).

The electron density clearly shows that the 2′-phosphate site remains occupied by an inorganic phosphate ion, as in the unliganded structure, allowing NADH to mimic NADPH (Figure 5). This possible mode of binding was already hypothesized by Worthington & Rosemeyer (1976) to explain the stimulatory effect of phosphate ion on the enzymatic activity when NADH was used as a substrate. Relative to the position of the 2′-phosphate of NADPH, the inorganic phosphate is pushed about 0.5 Å deeper into the enzyme. Even so, due to the presence of the noncovalently bound phosphate ion, the adenine and atoms C1′A and C2′A of the adenine ribose are about 0.5 Å further away from the surface of the enzyme than observed for NADPH. In addition, the adenine ribose of NADH assumes the 2′-endo conformation, which moves O2′A up and away from the inorganic phosphate. It seems likely that both O2′A and O3′A donate H-bonds to the inorganic phosphate (see Figure 5). O4′A is still in a position to accept a somewhat weaker H-bond from 195-N. The nicotinamide half of NADH binds in the same way as NADPH (Figure 5). Although the adenosine moiety and the inorganic phosphate are not optimally bound, the density suggests that their mobilities are comparable to those for bound NADPH. The difference in binding affinity is thus probably due to a number of small contributions rather than any single major unfavorable interaction dislocating a particular part of the ligand.

(*E*) *Binding of Fragments of NADPH.* The binding of fragments of NADPH has been studied for better characterization of the binding site. The 5.8-Å resolution analyses of fragments with no 2′-phosphate group (soaks T–W, Table II) indicate that none of these compounds binds properly. In each case, there is only a weak signal in the difference Fourier, indicating some binding in the hydrophobic pocket normally occupied by the adenine moiety. This emphasizes the central role of the 2′-phosphate for specific binding.

A series of fragments having the 2′-phosphate, from 2′-AMP through 2′P-5′ADPrib, were analyzed at 3-Å resolution (soaks D–I, Table I). All of these compounds act as competitive inhibitors of NADPH binding, and the difference maps in each case showed binding only to the NADPH site. The occupancies of the individual inhibitors at the 2′-phosphate site varied from a minimum of 27% for 2′P-5′ADP (soak F) to 100% for 2′P-5′AMP (soak E). The data are given in Table I. As shown by Pai et al. (1984), the observed occupancies correlate roughly with the respective binding constants. This supports the idea that the crystallographically observed binding modes accurately portray the mode of binding in solution. However, the observed occupancies are far below the saturation predicted on the basis of binding constants and soak concentrations (Table I). This could well be due to the high ionic strength of 6.0 of the solvent in the crystal.

The smallest fragment tested in this study was 2′AMP (soak D). The 2′-phosphate bound at the expected position, but the density for the adenine group is rather broad, suggesting that on the average it is somewhat displaced from the true binding site and is therefore less tightly held. Nevertheless, the binding of 2′AMP causes the conformational change of Arg-218. The addition of the 5′-phosphate dramatically improves binding, such that 2′P-5′AMP binds in the same way as the corresponding moiety in NADPH and with quite high occupancy (soak E, Table I). This gives a rationale for its use as an affinity ligand in the purification of the enzyme (Krohne-Ehrich et al., 1977). The improved binding can probably be best explained in that the 5′-phosphate provides a second fixed point on the ligand, greatly decreasing its degree of freedom. Without this group, only the 2′-phosphate is held at a specific place, while the adenine group, despite having its orientation fixed by being sandwiched between Ile-289 and Arg-218 (see Figure 3), is apparently somewhat free to undergo translations in that plane. The extra negative charge of the 5′-phosphate group is easily accommodated as it points out toward the solvent. Moreover, the total charge of the 5′-phosphate is most probably equal to that of the pyrophosphate moiety of NADP.

The addition of a third phosphoryl group to give 2′P-5′ADP makes the binding worse (soak F, Table I). In NADPH, this phosphoryl group points back toward the enzyme and is inaccessible to bulk solvent (Figure 3). The difference density of soak F indicates that for 2′P-5′ADP the terminal 5′-phosphoryl group points into the solvent (Table I). Presumably, it carries a surplus negative charge that is unacceptable at the N-phosphoryl position. Analogous behavior was observed for the binding of ADP to NAD-dependent L-lactate dehydrogenase (Chandrasekhar et al., 1973). The hypothesis that the extra charge disrupts binding is supported by the improvement in binding observed for 2′P-5′ADPme (soak G),

which has the proper charge at the pyrophosphate. In this case, the pyrophosphate binds at the correct position as defined by NADPH binding.

During the binding of 2′P-5′ADPrib (soaks H and I) a movement of Tyr-197 is observed. Due to steric effects, the ribose cannot bind without a movement of Tyr-197 and Ile-198. In these soaks the ligand binds with about 60% occupancy at the pyrophosphate, and the side chain of Tyr-197 seems to occupy its alternate position away from FAD for about 25% of the molecules (Table I). This suggests that the two conformations, "ribose-in/Tyr-out" and "Tyr-in/ribose out", are close in energy. Since the soaks with the oxidized (soak H) and the reduced (soak I) enzyme gave similar results, the oxidation state of the enzyme does not play a major role in the induced fit involving Tyr-197. The somewhat poorer binding observed for soak I can be explained by the lower concentration of ligand used in this soak (Table I).

(*F*) *NADP Analogues.* The binding behavior of analogues of NADP with a modified nicotinamide group (soaks K–M) or a modified adenine group (soak N) has also been studied. The oxidized forms of these analogues were used in the presence of a great excess of β-mercaptoethanol. All of these oxidized analogues should have a positive charge at the pyridine ring and thus should not bind well in the nicotinamide pocket. However, the ratios of the occupancies at Tyr-197-out and **PR** for these analogues (Table I) show that the conformational change involving Tyr-197 generally took place for a greater portion of the *liganded* molecules than was observed for either of the $NADP^+$ soaks.

One possible explanation is that the tighter binding is due to a certain amount of the uncharged reduced form that is produced by the back reaction. The series of experiments with NAD^+ at 6-Å resolution (soaks P–S, Table II) provides strong support for this explanation. A comparison of soaks P and Q establishes that the reduction state of the enzyme does not change the binding of NAD^+. Assuming the structural equivalence of EH_2 and EHR at the nicotinamide binding pocket, the observed binding to EH_2, the active form of the reduced enzyme (soak R), must be due to catalytically generated NADH. Although the reverse reaction is very slow (Icén, 1971), the presence of very high concentrations of the ligands and of enzyme in the crystal, coupled with the long times involved in the experiment (Table I), can lead to reduction of the modified nucleotides. Therefore, with the exception of $amNADP^+$ the observed binding can be explained by the presence of a mixture of the oxidized and reduced forms of the analogues. The analogues for which this ambiguity exists are designated xxNADP(*H*).

The compound $amNADP^+$ is a special case, as it is quite resistant to reduction (Anderson & Kaplan, 1959) and thus presumably binds in its oxidized, positively charged form. Despite its charge, $amNADP^+$ binds reasonably well in the nicotinamide pocket (soak K, Table I). The difference Fourier shows less density at the position normally occupied by the carboxamide group and is consistent with the presence of the smaller amine substituent. The electron-donating properties of this 3-amino group are strong (Anderson & Kaplan, 1959), such that it may remove a significant portion of the positive charge from the pyridine ring.

Among the other analogues thNADP(H), in which a sulfur atom replaces O7N (soak L), binds with the highest occupancy at the nicotinamide site. The form of the electron density due to thNADP(H) is indistinguishable from that of NADPH. Only the density at the presumed sulfur position is, as expected, significantly higher. The larger van der Waals radius of the sulfur appears to be accepted with minimal movement of neighboring atoms. This agrees with the observation that there are no direct hydrogen bonds from protein atoms to this position (see above). The occupancy of acNADP(H), which has N7N replaced with a methyl group (soak M), in the nicotinamide pocket is too low for a meaningful analysis of its conformation. In contrast to the less restrictive environment of O7N, N7N makes two hydrogen bonds to protein atoms (see above) such that a methyl group is discriminated against at this site. In agreement with its relatively poor inhibition constant, etNADP(H) bound very weakly (see Table I). On the basis of the structure of the binding site of the adenine moiety, however, it is not clear why this should be so. The N10A and N1A atoms of the adenine are quite exposed to solvent, leaving plenty of room for modifications at these atoms.

(*G*) *Conclusions.* The binding of NADPH and a number of related fragments to human glutathione reductase has been structurally investigated. The binding mode of NADPH has been described. A similar binding mode for NADH was observed, with an inorganic phosphate substituting for the 2′-phosphate of NADPH. The studies with fragments and analogues of NADPH highlight a number of essential features.

(1) The binding of these extended ligands was not an all or none phenomenon.

(2) The 2′P-5′AMP end of the molecule always bound the best, and compounds other than NADH that had no 2′-phosphate showed no binding.

(3) The binding was weakest for buried ligand parts with modified charged or polar groups. Particularly sensitive positions are the N-phosphoryl group and the pyridine ring and the amide-NH_2 group of the nicotinamide.

(4) The induced fit involving movement of Tyr-197 required a ligand extending from adenine to at least the N-ribose. It was independent of the reduction state of the enzyme. This suggests that the substrate NADPH itself may directly trigger the conformational change.

Taken together, these observations suggest that productive binding of NADPH begins with binding of the adenine end of the molecule to the preformed 2′-phosphate binding site. In this way, the nicotinamide gains the leverage needed to force the movement of Tyr-197 and enter into the pocket at the *re* face of the flavin.

ACKNOWLEDGMENTS

We thank Dr. R. Goody and M. Isakov, Heidelberg, for providing 2′P-5′ADP and 2′P-5′ADPme and Dr. C. H. Williams and D. Arscott, Ann Arbor, for $amNADP^+$. Coordinates for the NADPH-liganded enzyme will be deposited in the Protein Data Bank, Brookhaven, NY.

Registry No. NADPH, 53-57-6; 2′AMP, 130-49-4; 2′P-5′AMP, 3805-37-6; 2′P-5′ADP, 4457-01-6; 2′P-5′ADPme, 29388-55-4; 2′P-5′ADPrib, 4197-14-2; NADH, 58-68-4; amNADP, 54758-28-0; thNADP, 19254-05-8; acNADP, 341-67-3; etNADP, 56973-46-7; NADP, 53-59-8; 5′ADPrib, 20762-30-5; GSSG, 27025-41-8; FAD, 146-14-5; nicotinamide, 98-92-0; phosphate, 14265-44-2; glutathione reductase, 9001-48-3.

REFERENCES

Anderson, B. M., & Kaplan, N. O. (1959) *J. Biol. Chem. 234*, 1226–1232.

Arscott, L. D., Thorpe, C., & Williams, C. H., Jr. (1981) *Biochemistry 20*, 1513–1520.

Blundell, T. L., & Johnson, L. N. (1976) *Protein Crystallography*, Academic, London.

Brown, N. L., Ford, S. J., Pridmore, R. D., & Fritzinger, D. C. (1983) *Biochemistry 22*, 4089–4094.

Carlberg, I., & Mannervik, B. (1986) *J. Biol. Chem. 261*, 1629–1635.
Chandrasekhar, K., McPherson, A., Jr., Adams, M. J., & Rossmann, M. G. (1973) *J. Mol. Biol. 76*, 503–518.
Eklund, H., Samama, J. P., & Jones, T. A. (1984) *Biochemistry 23*, 5982–5996.
Feeney, J., Birdsall, B., Roberts, G. C. K., & Burgen, A. S. V. (1975) *Nature (London) 257*, 564–566.
Filman, D. J., Bolin, J. T., Matthews, D. A., & Kraut, J. (1982) *J. Biol. Chem. 257*, 13663–13672.
Fita, I., & Rossmann, M. G. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 1604–1608.
Fox, B. S., & Walsh, C. T. (1983) *Biochemistry 22*, 4082–4088.
Greer, S., & Perham, R. N. (1986) *Biochemistry 25*, 2736–2742.
Hol, W. G. J., Van Duijnen, P. Th., & Berendsen, H. J. C. (1978) *Nature (London) 273*, 443–446.
Icén, A. L. (1971) *FEBS Lett. 16*, 29–32.
Karplus, P. A., & Schulz, G. E. (1987) *J. Mol. Biol. 195*, 701–729.
Koshland, D. E., Jr. (1958) *Proc. Natl. Acad. Sci. U.S.A. 44*, 98–104.
Krauth-Siegel, R. L., Blatterspiel, R., Saleh, M., Schiltz, E., Schirmer, R. H., & Untucht-Grau, R. (1982) *Eur. J. Biochem. 121*, 259–267.
Krauth-Siegel, R. L., Anders, B., Henderson, G. B., Fairlamb, A. H., & Schirmer, R. H. (1987) *Eur. J. Biochem. 164*, 123–128.
Krohne-Ehrich, G., Schirmer, R. H., & Untucht-Grau, R. (1977) *Eur. J. Biochem. 80*, 65–71.
Laddaga, R. A., Chu, L., Misra, T. K., & Silver, S. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 5106–5110.
Manstein, D. J., Pai, E. F., Schopfer, L. M., & Massey, V. (1986) *Biochemistry 25*, 6807–6816.
Misra, T. K., Brown, N. L., Haberstroh, L., Schmidt, A., Godette, D., & Silver, S. (1985) *Gene 34*, 253–262.
Pai, E. F., & Schulz, G. E. (1983) *J. Biol. Chem. 258*, 1752–1757.
Pai, E. F., Horn, E., & Schulz, G. E. (1984) in *Flavins and Flavoproteins* (Bray, R. C., Engel, P. C., & Mayhew, S. G., Eds.) pp 139–142, de Gruyter, Berlin.
Rice, D. W., Schulz, G. E., & Guest, J. R. (1984) *J. Mol. Biol. 174*, 483–496.
Rossmann, M. G., Liljas, A., Brändén, C.-I., & Banaszak, L. J. (1975) *Enzymes (3rd Ed.) 11*, 61–102.
Schulz, G. E., Schirmer, R. H., & Pai, E. F. (1982) *J. Mol. Biol. 160*, 287–308.
Shames, S. L., Fairlamb, A. H., Cerami, A., & Walsh, C. T. (1986) *Biochemistry 25*, 3519–3526.
Stephens, P. E., Lewis, H. M., Darlison, M. G., & Guest, J. R. (1983) *Eur. J. Biochem. 135*, 519–527.
Stern, B. K., & Vennesland, B. (1960) *J. Biol. Chem. 235*, 209–212.
Thieme, R., Pai, E. F., Schirmer, R. H., & Schulz, G. E. (1981) *J. Mol. Biol. 152*, 763–782.
Tronrud, D. E., TenEyck, L. F., & Matthews, B. W. (1987) *Acta Crystallogr., Sect. A: Found. Crystallogr. A43*, 489–501.
Wierenga, R. K., Drenth, J., & Schulz, G. E. (1983) *J. Mol. Biol. 167*, 725–739.
Wierenga, R. K., De Maeyer, M. C. H., & Hol, W. G. J. (1985) *Biochemistry 24*, 1346–1357.
Williams, C. H., Jr. (1976) *Enzymes (3rd Ed.) 13*, 89–172.
Worthington, D. J., & Rosemeyer, M. A. (1976) *Eur. J. Biochem. 67*, 231–238.
Wright, W. B., & King, G. S. D. (1954) *Acta Crystallogr. 7*, 283–288.
Yanagawa, H. (1979) *Methods Enzymol. 62*, 172–181.