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Localization of cofactor binding sites with monoclonal anti-idiotype antibodies: Phenylalanine hydroxylase

(pterin cofactor/aromatic amino acid hydroxylases/active site/phenylalanine 4-monooxygenase)

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ABSTRACT **A monoclonal anti-idiotype antibody, NS7, previously shown to mimic the binding of the pterin cofactor of phenylalanine hydroxylase (phenylalanine 4-monooxygenase, EC 1.14.16.1) has been used to localize the cofactor binding site within the phenylalanine hydroxylase catalytic domain to a 27-amino-acid sequence that is highly conserved among the three aromatic amino acid hydroxylases. The binding of NS7 to a synthetic peptide corresponding to the phenylalanine hydroxylase sequence from residue 263 to residue 289 was blocked by the competitive inhibitor of phenylalanine hydroxylase enzyme activity, 7,8-dihydro-6,7-dimethylpterin. In addition this peptide competed with native phenylalanine hydroxylase for binding to 6,7-dimethyl-5,6,7,8-tetrahydropterin conjugated to a polyglutamate carrier. Application of this simple and direct approach to other enzymes is likely to greatly facilitate the identification of ligand binding sites on enzymes, which will significantly contribute to the understanding of enzyme structure–function relationships.**

There are many methods available for defining the regions within enzymes responsible for binding their substrates (1). The most definitive method is determination of the three-dimensional structure of the enzyme–substrate complexes by x-ray crystallography. However, this method tends to be time-consuming, specialized, and by no means routine or certain. Reactive substrate analogues require specialized chemistry for their synthesis and are not commonly available for all enzymes, and large substitutions on substrate molecules can lead to binding with amino acids away from the active site. Selective chemical modification of amino acids has been useful, but conformational and steric effects generated by the modified amino acids can produce misleading results. *In vitro* mutagenesis has been used increasingly in recent times, but some prior information on the structure–function relationship of the enzyme is required in order to focus on the amino acids to be altered, and again indirect effects of distant changes can affect the active site of an enzyme. Here we have assessed the utility of the anti-idiotype antibody approach in the identification of the cofactor binding site in the phenylalanine hydroxylase (PH; phenylalanine 4-monooxygenase, EC 1.14.16.1) catalytic site. PH converts phenylalanine to tyrosine by using a reduced pterin cofactor (tetrahydrobiopterin), enzyme-bound iron, and oxygen (2). Although the linear amino acid sequence of PH is known (3, 4), little is known of the structure of the enzyme active site or that of the other aromatic amino acid hydroxylases. In the inherited metabolic disease phenylketonuria, PH enzyme activity has been shown to be deficient (5). Identification of PH mutations, determining the sequence, and subsequent expression of the mutant PH in bacteria has not as yet revealed any information about the PH active site as reviewed in ref. 6. Thiol modification (7), phosphorylation (8), the addition of lysolecithin, and limited chymotryptic digestion (9) all affect PH enzyme activity but do not apparently act directly on the active site.

To develop a probe for the PH catalytic site, we have previously isolated a monoclonal auto-anti-idiotype antibody, NS7, from a mouse immunized with 6,7-dimethyltetrahydropterin (10) by using a method described by Cleveland *et al.* (11). Based on several lines of evidence, the NS7 monoclonal antibody was proposed to mimic the reduced pterin, as predicted by the network theory of immune regulation proposed by Jerne (12), by binding to the pterin cofactor binding site on PH and several other pterin-utilizing enzymes (10, 13). Isolation and characterization of the NS7 antibody have now enabled its use as a structural probe for the localization of both the NS7 epitope on PH and by analogy the pterin binding site on PH.

MATERIALS AND METHODS

V8 protease (endoproteinase Glu-C) purified from *Staphylococcus aureus* was purchased from Miles. 7,8-Dihydro-6,7-dimethylpterin ($DMPH_2$) was a gift from W. Pfleiderer (Konstanz, F.R.G.).

Production and Characterization of PH Fragments. Rat liver PH was purified according to Choo *et al.* (14). Chymotryptic fragments were produced following activation of PH in 10 mM phenylalanine/10 mM Tris·HCl, pH 7.6 at 25°C for 10 min, after which chymotrypsin was added (enzyme/PH, 1:5) for 30 min at 30°C; digestion was stopped by the addition of an equal volume of sample buffer [20 mM Tris·HCl, pH 7.6, 4% (wt/vol) SDS/10% (vol/vol) glycerol/5% (vol/vol) 2-mercaptoethanol/0.01% (wt/vol) bromophenol blue] and incubation at 100°C for 5 min. Where enzyme activity was to be measured, the digestion was stopped by the removal of the chymotrypsin by lima bean chymotrypsin inhibitor linked to agarose (Sigma). The digest was then separated on a Mono Q column (Pharmacia) equilibrated with 30 mM Tris·HCl, pH 7.6, by using a 0–0.5 M NaCl gradient. Fractions were collected and assayed for PH enzyme activity (14). Active fractions were then analyzed by SDS/PAGE to identify the molecular masses of the active fragments. V8 protease fragments were produced by the incubation of PH in 50 mM ammonium bicarbonate (pH 8) with V8 protease (enzyme/PH, 1:5) for 24 h at 37°C. Digestion was then stopped as described above.

Proteolytic digests were then subjected to electrophoresis on a polyacrylamide gel containing 0.1% (wt/vol) SDS (15). Fragments were visualized by negative staining with 4 M sodium acetate (16); appropriate gel pieces were cut out and electroeluted at 10 mA for 16 h in 50 mM NH_4HCO_3. Protein concentrations of purified PH fragments were estimated by

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Abbreviations: PH, phenylalanine hydroxylase; $DMPH_2$, 7,8-dihydro-6,7-dimethylpterin.

using the method of Bradford (17). Cyanogen bromide (CNBr) digestion of purified fragments was carried out by mixing an equal volume of fragment [1 mg/ml in 70% (vol/vol) formic acid] and CNBr (50 mg/ml in 70% formic acid) for 16 h at 25°C under N_2. This digest was then air-dried, redissolved in 10 mM Tris·HCl, pH 7.6/2% SDS/5% glycerol/5% 2-mercaptoethanol/0.01% bromophenol blue, and heated at 100°C prior to electrophoresis. Resulting fragments were detected following SDS/PAGE either by silver staining (18) or immunoblotting using the previously isolated PH8 monoclonal antibody to PH (19). Molecular mass estimations of PH fragments were performed by using proteins of known molecular masses and the method of Weber and Osborn (20). The following proteins of known molecular masses were used as standards: bovine albumin (67 kDa), ovalbumin (43.0 kDa), bovine carbonic anhydrase (30.0 kDa), soybean trypsin inhibitor (20.1 kDa), horse heart myoglobin (16.9 kDa), and the 14.4-, 8.2-, and 6.2-kDa cyanogen bromide-cleaved fragments of myoglobin. From the molecular mass estimation of a fragment, the number of amino acids contained in the fragment was calculated by using an average molecular mass of 110 kDa. NS7 antibody immunoreactivity was estimated by coating 5 pmol of antigen onto plastic wells and by using an ELISA (10).

Characterization of NS7 Antibody Binding to the Peptide PH-(263–289). A peptide corresponding to the PH sequence between residues 263 and 289 [PH-(263–289)] was synthesized and purified as described (21). Purity was confirmed by high-pressure liquid chromatography on an Aquapore RP-300 column using a linear gradient (10–50%) of acetonitrile in 0.1% trifluoroacetic acid. Negative control peptides P8 (sequence Glu-Gly-Leu-Phe-Asp-Lys-Gly-Leu-Phe-Asp) and KT (sequence Leu-Arg-Arg-Ala-Ser-Leu-Gly) were obtained commercially, and PH-(10–30), corresponding to amino acids 10–30 on the PH sequence, was produced as detailed above.

NS7 immunoreactivity was estimated by coating plastic wells with peptide (5 μg/ml in 0.05 M $NaHCO_3/Na_2CO_3$ at pH 9.6) and using the previously described ELISA (10). NS7 immunoreactivity with peptides in solution was measured by a competitive ELISA such that NS7 antibody (at a 1:50 dilution) was preincubated with various amounts of peptide for 1 h at 4°C prior to addition of this mixture to wells coated with PH and estimation of the amount of bound antibody by the ELISA method.

Pterin binding by peptides was assayed in two different ways. (*i*) PH-(263–289) peptide-coated plastic wells were incubated with various concentrations of $DMPH_2$ followed by addition of NS7 antibody (1:50 dilution) and estimation of the amount of antibody bound by ELISA. (*ii*) 6,7-Dimethyl-5,6,7,8-tetrahydropterin conjugated to a polyglutamate carrier using carbodiimide (10) was coated onto plastic wells (10 μg/ml in 0.05 M $NaHCO_3$ at pH 9.6). Wells were washed with 0.1 M Na_2HPO_4/NaH_2PO_4, pH 7.4/0.15 M NaCl and then incubated with various amounts of peptide PH-(263–289) or a negative control peptide, P8, followed by addition of 1.25 nmol of PH in 1% bovine albumin/0.1 M Na_2HPO_4/NaH_2PO_4, pH 7.4/0.15 M NaCl. The amount of PH bound to the pterin was quantitated by using the PH8 monoclonal antibody and the ELISA method.

RESULTS AND DISCUSSION

In an ELISA the NS7 antibody bound to PH denatured by boiling but not to PH when binding was tested by immunoblotting, indicating that although the epitope appeared to be linear immunoblotting would not be of use in localization of the epitope (results not shown). Complete proteolytic digestion of PH with either trypsin, chymotrypsin, or V8 protease did not yield any peptides immunoreactive with NS7. Therefore fragments of PH generated by limited proteolysis were isolated in order to localize the NS7 immunoreactive fragment.

Limited chymotryptic digestion of PH gave rise to two major fragments of 38 and 31 kDa (Fig. 1*A*). Separation of these two fragments by anion-exchange chromatography showed that both of these fragments retained PH enzyme activity (results not shown). The 31-kDa chymotryptic PH fragment (CD_{31}) was purified by electroelution and analyzed by SDS/PAGE (Fig. 1*B*). Sequence localization was determined by CNBr digestion of the CD_{31} fragment at the single methionine, amino acid 275 (Met-275), on the rat PH sequence

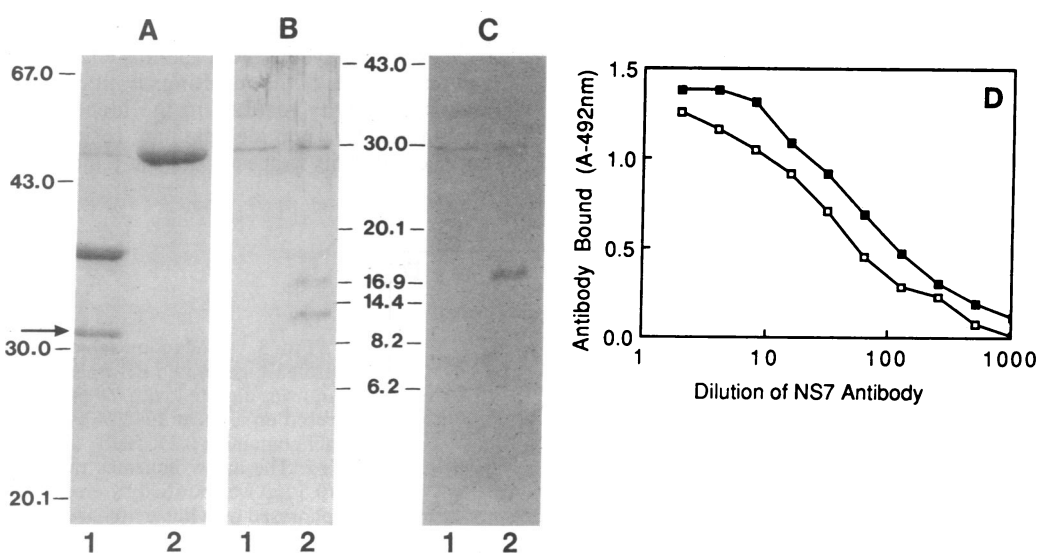

FIG. 1. Production and characterization of PH chymotryptic fragment CD_{31}. (*A*) Coomassie blue stain of a 12% polyacrylamide gel containing 0.1% SDS. Lane 1, 15 μg of PH digested with chymotrypsin. The arrow indicates the position of CD_{31}. Lane 2, 10 μg of PH alone. (*B*) Silver stain of a linear 10–20% gradient polyacrylamide gel containing 0.1% SDS. Lane 1, 0.5 μg of CD_{31} purified by electroelution; lane 2, 3 μg of purified CD_{31} cleaved with CNBr. (*C*) Western blot, using the PH8 monoclonal antibody, of CD_{31} with (lane 2) and without (lane 1) CNBr cleavage. The positions on the gel of proteins of known molecular mass (in kDa) are indicated. (*D*) Five picomoles of PH (■) and CD_{31} (□) were coated onto plastic wells, and NS7 antibody immunoreactivity was quantitated by ELISA. Titration curves of the amount of NS7 antibody bound at various dilutions are shown. Results have been corrected for nonspecific binding measured by using an IgM monoclonal antibody that does not bind PH.

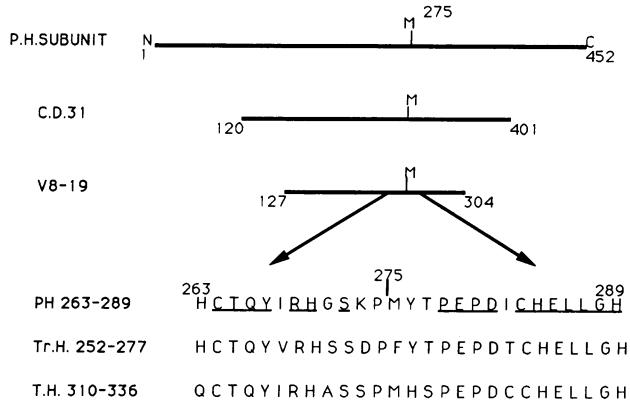

FIG. 2. NS7 epitope on PH. A linear representation of the sequence of the NS7 immunoreactive PH fragments and the amino acid sequence of the immunoreactive peptide PH-(263–289) are shown. Amino acids in PH-(263–289) conserved among the three aromatic amino acid hydroxylases are underlined. The homologous sequences in tryptophan hydroxylase (Tr.H. 252–277) and tyrosine hydroxylase (T.H. 310–336) are also shown.

(4). The size of the fragment, N-terminal of Met-275, was determined by immunoblotting with the PH8 monoclonal antibody whose PH epitope has been characterized as amino acids 139–155 on the PH sequence (19). CNBr digestion of CD_{31} yielded two fragments of estimated molecular mass of 17.3 and 13.7 kDa (Fig. 1*B*) of which the 17.3-kDa CNBr fragment was shown to be N-terminal of Met-275 due to its PH8 immunoreactivity (Fig. 1*C*). From these molecular mass determinations, CD_{31} was estimated to span from Phe-120 to Tyr-401 on the PH sequence (Fig. 2). NS7 showed comparable immunoreactivity towards CD_{31} compared to the whole PH molecule (Fig. 1*D*). This fragment thus retains enzyme activity and the ability to bind the pterin-mimicking antibody NS7.

A V8 protease digest of PH produced a fragment ($V8_{19}$) of 19 kDa (Fig. 3*A*) that did not retain enzyme activity (results not shown). This fragment was also purified by SDS/PAGE and electroelution (Fig. 3*B*) and found to be immunoreactive with PH8 antibody (Fig. 3*C*). The $V8_{19}$ fragment was estimated to span amino acids Leu-127 to Glu-304 on the PH amino acid sequence (Fig. 2) because digestion with CNBr decreased the molecular mass of the PH8 immunoreactive fragment by 2.2 kDa (Fig. 3*C*), indicating the loss of ≈20 amino acids from the C-terminal side of Met-275 with the nearest V8 protease cleavage site being at Glu-304. NS7, the pterin-mimicking antibody, was found to bind to $V8_{19}$ in an ELISA comparable to NS7 binding to whole PH (Fig. 3*D*). Although $V8_{19}$ does not retain enzyme activity, NS7 still reacts, indicating that the pterin binding site is still intact although other parts of the catalytic site may have been destroyed.

Further reduction in the size of the NS7 immunoreactive fragment on PH was achieved by using a 27-amino-acid synthetic peptide, PH-(263–289) (Fig. 2). The peptide PH-(263–289) was chosen because it is highly conserved among the aromatic amino acid hydroxylases, is located within the sequence of $V8_{19}$, the previously identified NS7 immunoreactive fragment, and contains a high proportion of potential metal binding ligands. The significance of the latter property is not clear, although previous work on the mechanism of the prereduction step has shown electron transfer from the reduced pterin to Fe^{3+} bound to PH, suggesting that the iron and pterin are in close proximity to each other in the catalytic site (22). The peptide PH-(263–289) was found to bind to NS7 antibody, whereas peptide PH-(10–30) was not immunoreactive with NS7 antibody (Fig. 4*A*). The peptide PH-(263–289) was found to block completely the binding of NS7 antibody to intact PH subunit (Fig. 4*B*), indicating that the entire NS7 epitope is located within this region. Negative control peptides showed no competitive binding. Previously $DMPH_2$ was shown to inhibit NS7 binding to PH in an ELISA (10). $DMPH_2$, at a concentration of 100 μM, has been shown to increase the K_m of 6-methyl-5,6,7,8-tetrahydropterin for PH from 25.0 to 39.0 μM but to have no effect on the V_{max}, indicating that $DMPH_2$ is a competitive inhibitor of PH with respect to the pterin cofactor binding site of PH. It was found that NS7 binding to PH-(263–289) was inhibited by increasing concentrations of $DMPH_2$ (Fig. 4*C*). The concentration of $DMPH_2$ required for half-maximum inhibition of binding was 0.37 mM, which is comparable to the value of 0.45 mM previously determined for the intact PH subunit (10). PH-(263–289) peptide was shown to specifically compete with native PH for binding to 6,7-dimethyl-5,6,7,8-tetrahydropterin conjugated to polyglutamate (Fig. 4*D*); this provides evidence, independent of NS7 antibody binding, that PH-(263–289) peptide does bind pterin.

Proteolytic digestions of the PH-(263–289) peptide with trypsin, thermolysin, endoproteinase (Arg), and V8 protease and purification of the resulting peptides and testing for NS7 immunoreactivity resulted in the identification of only one immunoreactive peptide spanning residues 263–286 (results not shown). This suggests that the majority of the peptide is

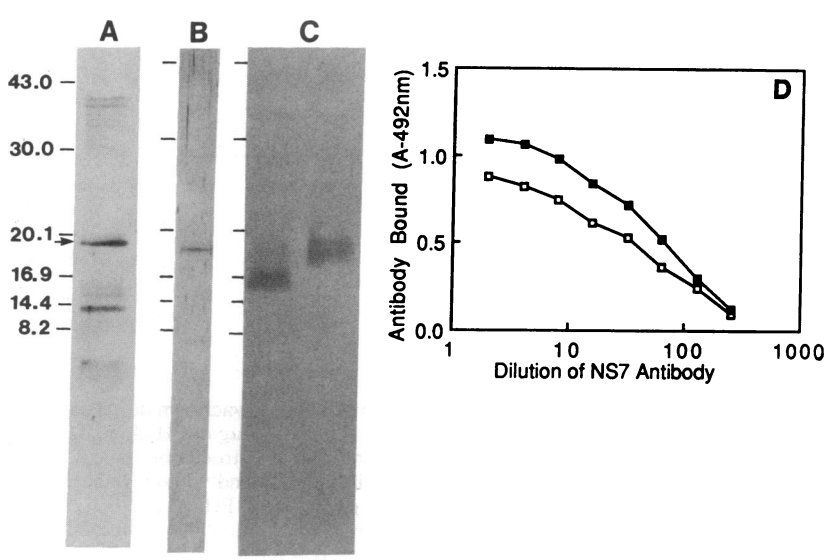

FIG. 3. Production and characterization of a V8 protease-generated PH fragment, $V8_{19}$. (*A*) PH (15 μg) was digested with V8 protease and electrophoresed on a linear 10–20% polyacrylamide gradient gel containing 0.1% SDS, followed by silver staining. The arrow indicates the $V8_{19}$ band. (*B*) $V8_{19}$ (0.5 μg) was purified by electroelution and electrophoresed on a linear 10–20% gradient of polyacrylamide containing 0.1% SDS, followed by silver staining. (*C*) A Western blot, using the PH8 antibody, of a linear 10–20% polyacrylamide gradient gel containing 0.1% SDS is shown. Purified $V8_{19}$ treated with CNBr (lane 1) and the untreated control (lane 2) are shown. The positions on the gel of proteins of known molecular masses (in kDa) are indicated. (*D*) Five picomoles of PH (■) and $V8_{19}$ (□) were coated onto plastic wells, and NS7 antibody immunoreactivity was quantitated by ELISA (10).

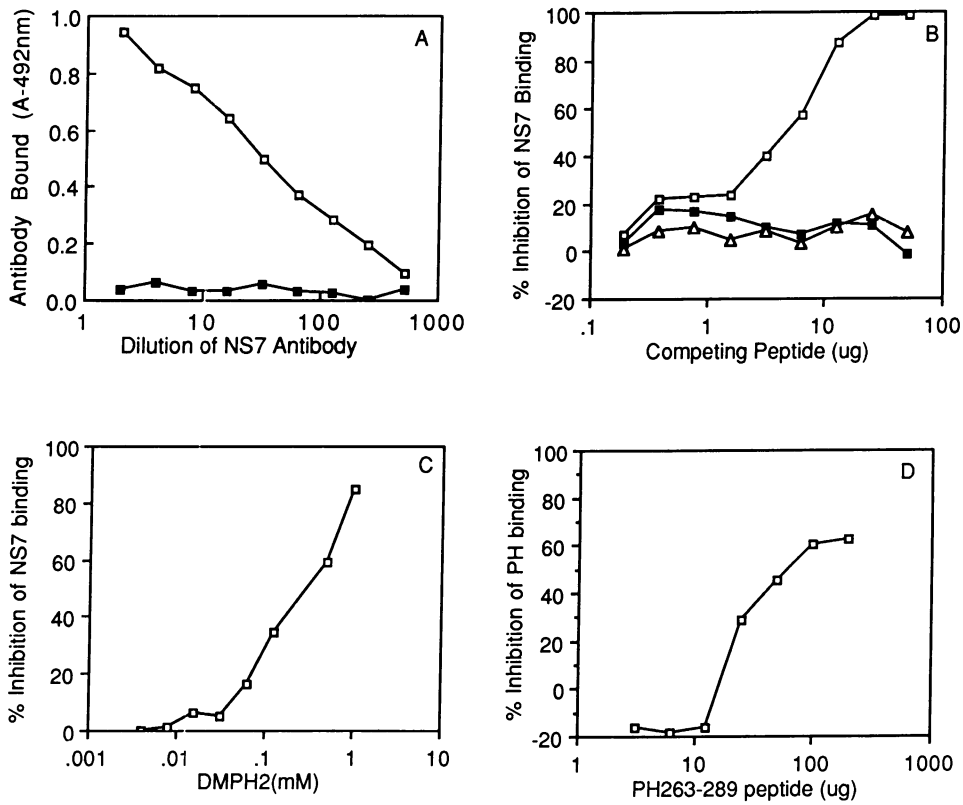

FIG. 4. Characterization of the synthetic peptide [PH-(263–289)]. (*A*) Peptides (5 μg/ml) were coated onto plastic wells and NS7 immunoreactivity was measured by ELISA. The amount of NS7 antibody bound to the peptide PH-(263–289) (□) and the PH-(10–30) peptide (■) is shown at various dilutions of NS7 antibody added. (*B*) Results of competitive ELISA showing the effect of adding increasing amounts of peptide PH-(263–289) (□), KT (■), and P8 (△) to a 1:50 dilution of NS7 antibody, followed by addition of this mixture to PH-coated plastic wells. The percent inhibition of NS7 binding was determined by using the amount of antibody bound in the absence of peptide as 100% binding. (*C*) Results of ELISA showing the effect of increasing concentrations of $DMPH_2$ on the binding of NS7 antibody (1:50 dilution) to PH-(263–289). The percent inhibition of NS7 binding was determined by using the amount of antibody bound in the absence of pterin as 100% binding. (*D*) 6,7-Dimethyl-5,6,7,8-tetrahydropterin conjugated to a polyglutamate carrier using carbodiimide (10) was coated onto plastic wells, and the ability of peptide PH-(263–289) to compete with PH for binding to the pterin was quantitated by using PH8 monoclonal antibody and the ELISA procedure. The percent inhibition of PH binding was calculated by using the amount of PH bound in the presence of P8 peptide as 100%.

required for NS7 immunoreactivity and, by analogy, for pterin binding.

Here we have used a monoclonal anti-idiotype antibody to PH produced by immunization with a PH pterin cofactor (10) to locate a 27-amino-acid segment of PH that comprises part or all of the pterin cofactor binding site of PH. The high sequence homology of this region in PH with residues 252–278 of tryptophan hydroxylase and residues 310–326 of tyrosine hydroxylase (23) strongly suggests that these regions also act as pterin binding sites. It is clear that anti-idiotype antibodies have the potential to be used as simple and powerful tools in locating ligand binding sites for substrates, cofactors and regulating ligands on other enzymes without distortion of the native state of the enzyme, and by their unique property of mimicking a small molecule anti-idiotype antibodies avoid identification of residues near the binding site, as has occurred when reactive substrate analogues have been used. Indeed it may be possible to extend the approach to the identification of the binding sites for segments of enzymes that have important regulatory functions such as pseudosubstrate protein kinase prototopes (24). In these ways, the use of anti-idiotype antibodies to map the catalytic, regulatory, and other ligand binding sites on enzymes is likely to greatly accelerate the development of concepts of their structure–function relationships.

1. Kraut, J. (1988) *Science* **242**, 533–540.
2. Fisher, D. B., Kirkwood, R. & Kaufman, S. (1972) *J. Biol. Chem.* **247**, 5161–5167.
3. Kwok, S. C. M., Ledley, F. D., DiLella, A. G., Robson, K. J. H. & Woo, S. L. C. (1985) *Biochemistry* **24**, 556–561.
4. Dahl, H.-H. M. & Mercer, J. F. B. (1986) *J. Biol. Chem.* **261**, 4148–4153.
5. Scriver, C. R., Kaufman, S. & Woo, S. L. C. (1989) in *The Metabolic Basis of Inherited Disease*, eds. Scriver, C. R., Beaudet, A. L., Sly, W. S. & Valle, D. (McGraw-Hill, New York), Vol. 1, pp. 455–546.
6. Cotton, R. G. H. (1990) *J. Inherited Metab. Dis.* **13**, 739–750.
7. Parniak, M. A. & Kaufman, S. (1981) *J. Biol. Chem.* **256**, 6876–6882.
8. Milstein, S., Abita, J.-P., Chang, N. & Kaufman, S. (1976) *Proc. Natl. Acad. Sci. USA* **73**, 1591–1593.
9. Fisher, D. B. & Kaufman, S. (1973) *J. Biol. Chem.* **248**, 4345–4353.
10. Jennings, I. G. & Cotton, R. G. H. (1990) *J. Biol. Chem.* **265**, 1885–1889.
11. Cleveland, W. L., Wasserman, N. H., Sarangarajan, R., Penn, A. S. & Erlanger, B. F. (1983) *Nature (London)* **305**, 56–57.
12. Jerne, N. K. (1974) *Ann. Inst. Pasteur (Paris)* **125**, 373–389.
13. Ratnam, S., Ratnam, M., Cotton, R. G. H., Jennings, I. G. & Freisheim, J. H. (1989) *Arch. Biochem. Biophys.* **275**, 344–353.
14. Choo, K. H., Jennings, I. G. & Cotton, R. G. H. (1981) *Biochem. J.* **199**, 527–535.
15. Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685.
16. Higgins, R. C. & Dahmus, M. E. (1979) *Anal. Biochem.* **93**, 257–260.
17. Bradford, M. (1976) *Anal. Biochem.* **72**, 248–254.
18. Morissey, J. H. (1981) *Anal. Biochem.* **117**, 307–310.
19. Cotton, R. G. H., McAdam, W., Jennings, I. G. & Morgan, F. J. (1988) *Biochem. J.* **255**, 193–196.

20. Weber, K. & Osborn, M. (1969) *J. Biol. Chem.* **244,** 4406–4412.
21. Smith, S. C., McAdam, W. J., Kemp, B. E., Morgan, F. J. & Cotton, R. G. H. (1987) *Biochem. J.* **244,** 625–631.
22. Wallick, D. E., Bloom, L. M., Gaffney, B. J. & Benkovic, S. J. (1984) *Biochemistry* **23,** 1295–1302.
23. Grennet, H. E., Ledley, F. D., Reed, L. L. & Woo, S. L. C. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 5530–5534.
24. House, C. & Kemp, B. E. (1987) *Science* **238,** 1726–1728.