Characterization of a Phagocyte Cytochrome b_{558} 91-Kilodalton Subunit Functional Domain: Identification of Peptide Sequence and Amino Acids Essential for Activity†

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ABSTRACT: The phagocyte NADPH oxidase is a multicomponent membrane-bound electron transport chain that catalyzes the reduction of O_2 to superoxide. Cytochrome b_{558}, the terminal electron donor to O_2, is an integral membrane heterodimer containing 91- and 22-kDa subunits (gp91-*phox* and p22-*phox*, respectively). Synthetic peptides, whose amino acid sequences correspond to a gp91-*phox* carboxyl-terminal domain, inhibit superoxide production by blocking assembly of the oxidase from membrane and cytosol components. In this study, we examined the amino acid sequence requirements of a series of synthetic truncated gp91-*phox* peptides for inhibition of human neutrophil NADPH oxidase activation. RGVHFIF, corresponding to gp91-*phox* residues 559–565, was the minimum sequence capable of inhibiting superoxide generation. Contributions of individual amino acids to overall RGVHFIF inhibitory activity were determined by comparing the abilities of alanine-substituted RGVHFIF peptides to inhibit superoxide production. Substitution of alanine for arginine, valine, isoleucine, or either of the phenylalanines (but not glycine or histidine) within RGVHFIF resulted in loss of inhibitory activity. Synthetic gp91-*phox* carboxyl-terminal peptides are likely to be competitive inhibitors of the corresponding carboxyl-terminal domain of native gp91-*phox* by virtue of amino acid identity. We conclude that properties of arginine valine, isoleucine, and phenylalanine side chains within an RGVHFIF-containing domain of gp91-*phox* contribute significantly to cytochrome b_{558}-mediated activation of the oxidase.

Activation of neutrophils and other phagocytic cells by a variety of stimuli leads to a burst of cellular oxygen consumption and generation of superoxide (Rossi, 1986). A membrane-bound multicomponent oxidase catalyzes transfer of electrons from NADPH to oxygen, forming superoxide. Stimulation of neutrophils leads to assembly of the NADPH oxidase from cytosol and membrane proteins (Segal, 1989). The amino acid sequences of several of these NADPH oxidase components have been deduced. These components include cytosolic proteins of 47 and 67 kDa (p47-*phox* and p67-*phox*, respectively) and an integral membrane cytochrome b_{558} (Lomax et al., 1989; Volpp et al., 1989; Leto et al., 1990; Parkos et al., 1988; Royer-Pokora et al., 1986; Teahan et al., 1987). Cytochrome b_{558}, the terminal electron donor of the oxidase chain, is a glycosylated heterodimer with subunits of 91 and 22 kDa (gp91-*phox* and p22-*phox*) (Teahan et al., 1987; Parkos et al., 1987; Harper et al., 1985; Kleinberg et al., 1989). gp91-*phox* spans the membrane and its carboxy terminus is located on the cytoplasmic face of the membrane (Rotrosen et al., 1990).

Stimulation of normal neutrophils results in translocation of p47-*phox* and p67-*phox* from cytosol to membrane (Clark et al., 1990; Heyworth et al., 1991). In contrast to normal neutrophils, neither p47-*phox* nor p67-*phox* translocates from cytosol to membrane after stimulation of neutrophils from those chronic granulomatous disease patients whose membranes fail to express cytochrome b_{558} (Heyworth et al., 1991). This observation suggests that cytochrome b_{558} participates in the orderly assembly of NADPH oxidase from cytosol and membrane components in addition to transferring electrons in the fully formed oxidase.

Because of its intracellular location, we hypothesized that the carboxyl terminus of gp91-*phox* may interact with one or more cytosolic oxidase components, thereby facilitating formation of the oxidase. Antibodies directed against the carboxyl-terminal 13 amino acids of gp91-*phox* (residues 558–570) inhibited superoxide formation in a cell-free assay of neutrophil NADPH oxidase activity (Rotrosen et al., 1990). Synthetic peptides, whose amino acid sequences corresponded to the same gp91-*phox* carboxyl-terminal domain, specifically inhibited superoxide generation in the cell-free assay and in permeabilized neutrophils stimulated by phorbol myristate acetate or formylmethionylleucylphenylalanine (Rotrosen et al., 1990). We hypothesized that synthetic gp91-*phox* carboxyl-terminal peptides inhibited superoxide generation by blocking formation of the oxidase (Kleinberg et al., 1990). In the present study, we characterized the structural and functional requirements for inhibition of oxidase activation by gp91-*phox* carboxyl-terminal peptides.

Materials and Methods

Cell-Free Assay. Neutrophils were isolated from normal donor granulocyte pheresis packs (Kleinberg et al., 1990). Neutrophil membrane and cytosol fractions were prepared as described (Kleinberg et al., 1990) except that membrane was

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separated from cytosol by centrifugation at 48000g for 1 h. The cell-free assay for superoxide production was performed in 96-well microtiter plates with a final volume of 100 μL/well as described (Kleinberg et al., 1990). Membrane [0.5 μg of protein/well (2.6×10^5 cell equiv/well)] containing 0.7 pmol of cytochrome b_{558}/μg of protein was solubilized with deoxycholate as described (final deoxycholate:membrane protein weight ratio of 16:1) before addition to the cell-free assay (Kleinberg et al., 1990). Deoxycholate-solubilized membrane and synthetic gp91-*phox* carboxyl-terminal peptides were added to individual wells before activation of the cell-free assay by subsequent addition of reaction mixture (Nunoi et al., 1988) containing cytosol [16 μg of protein/well (5×10^5 cell equiv/well)] and arachidonate (30 μM final concentration). The maximal rate of superoxide generation (V_{max})[1] was quantitated as the maximal rate of superoxide dismutase-inhibitable reduction of acetylated ferricytochrome c measured at 550 nm on a Molecular Devices (Menlo Park, CA) Thermomax kinetic microtiter plate reader using 1-nm bandwidth filter (Kleinberg et al., 1990). Maximal ΔA_{550} was calculated by linear least-squares fit. V_{max} generally varied from 7 to 12 mODU/min in most experiments. Membrane and cytosol protein concentrations were measured with a microtiter plate bicinchoninic acid assay using bovine serum albumin as the protein standard according to manufacturer's instructions (Pierce, Rockford, IL).

Results reported in this paper were derived from dose/response experiments of gp91-*phox* carboxyl-terminal peptide concentration versus inhibition of superoxide production. Individual experiments were performed by adding various amounts of synthetic peptide spanning the complete concentration range of interest to duplicate wells on a single 96-well plate. Peptide solution diluent was added to control wells.

Synthetic Peptides. Peptides were synthesized by the Biological Resources Branch, NIAID, NIH, and purified by reverse-phase high-performance liquid chromatography (HPLC)[1] with exceptions noted below. The amino acid sequence was verified and peptide purity determined by automated peptide sequencing and HPLC. HPLC-purified peptides were >99% pure as measured by the relative amounts of contaminant amino acids at each peptide sequencing step. The only exceptions were two peptides, ESGPRGVHFIFNKENF and RGVHFIFNKENF, which were not purified by reverse-phase HPLC after synthesis and were 69% and 60% pure, respectively, with amino acid sequence and peptide purity determined as above. Major contaminants of both of these peptides consisted of short truncated peptides which did not contain the complete RGVHFIF sequence. Synthetic peptides were stored as lyophilized powders at −20 °C or as stock solutions in water at −70 °C. RGVAFIF was insoluble in water and was dissolved in dimethyl sulfoxide and stored in 5% dimethyl sulfoxide. When this peptide was used, the final dimethyl sulfoxide concentration in the cell-free assay was 0.6%, which, in the absence of peptide, did not affect NADPH oxidase activity. Concentrations of peptide stock solutions were determined by quantitative amino acid compositional analysis (Pico-Tag method, Waters, Bedford, MA) after 24-h hydrolysis in 6 N HCl according to manufacturer's instructions.

Data Analysis. V_{max} values of duplicate wells were averaged. Data were expressed as percent inhibition defined as (Rotrosen et al., 1990)

$$\% \text{ inhibition} = 100\{[V_{max(control)} - V_{max(peptide)}]/V_{max(control)}\} \quad (1)$$

where $V_{max(peptide)}$ and $V_{max(control)}$ were the maximal rates of superoxide production in the presence and absence of synthetic peptide, respectively. Means and standard errors of percent inhibition at each peptide concentration were calculated from replicate experiments and plotted as percent inhibition versus log peptide concentration (see Figure 1).

IC_{50} was defined as the peptide concentration that resulted in 50% inhibition of V_{max} compared to diluent controls without peptide. IC_{50} was used to quantitate the ability of a given peptide to inhibit NADPH oxidase activity. For inhibitory peptides (IC_{50} <300 μM), there was a sigmoidal dependence of percent inhibition of superoxide production on peptide concentration. We modeled this sigmoidal relationship with a modified Hill equation in order to determine IC_{50} precisely (Nakahiro et al., 1991; Ozawa et al., 1991). The Hill equation is

$$\% \text{ inhibition} = 100\{[\text{peptide}]^n/([\text{peptide}]^n + IC_{50}^n) + K\} \quad (2)$$

where K is a constant that accounts for error in zero peptide concentration values. Values of n and K were varied to optimize fit to percent inhibition/peptide concentration data by nonlinear regression (see Figure 1). Use of eq 2 markedly simplified determination of IC_{50} since IC_{50} was explicitly defined by eq 2. Data were explicitly weighted using standard errors calculated from replicate experiments, and goodness of fit was verified by examination of weighted residual plots. Correlation coefficients were 0.96 or better. For poorly inhibitory peptides, IC_{50} was not determined by Eq 2, but rather, a range of peptide concentrations that included IC_{50} was measured.

We chose to compare the relative inhibitory activities of gp91-*phox* peptides by measurements of IC_{50}'s instead of deriving Michaelis–Menten inhibition constants (K_i) for each peptide. Measurement of K_i offers the theoretical advantage over IC_{50} of independence of values of K_i from concentrations of oxidase components and levels of superoxide generating activity in the cell-free assay. However, determining K_i is inherently difficult in multicomponent, multistep processes such as NAPDH oxidase formation because derivation of K_i requires detailed knowledge of the kinetic mechanism for each step leading to assembly of the oxidase. Because current understanding of the mechanism of oxidase formation is incomplete, derivations of K_i were not feasible in our study.

Results

To determine the minimum peptide sequence capable of inhibiting NADPH oxidase activity, we synthesized a series of truncated gp91-*phox* carboxyl-terminal peptides and tested their abilities to inhibit superoxide generation in the cell-free assay. Synthetic gp91-*phox* carboxyl-terminal peptides likely share common physical and structural properties with the native gp91-*phox* carboxyl terminus because of amino acid sequence identity between the synthetic peptides and matching gp91-*phox* carboxy-terminal domain. This suggests that synthetic gp91-*phox* carboxy-terminal peptides may competitively inhibit native gp91-*phox* carboxyl-terminus participation in a reaction leading to formation of the NADPH oxidase. Previously described inhibitory gp91-*phox* carboxyl-terminal peptides include SNSESGPRGVHFIFNKENF (gp91-*phox* residues 552–570), RGVHFIFNKENF (residues 559–570), and RGVHFIF (residues 559–565) (Rotrosen et

[1] Abbreviations: HPLC, high-performance liquid chromatography; V_{max}, maximal rate of superoxide generation; IC_{50}, concentration of peptide that inhibited 50% of superoxide generating activity; K_i, Michaelis–Menten inhibition constant.

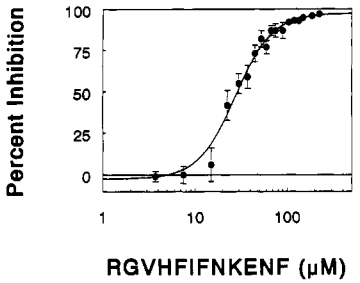

FIGURE 1: Dose/response relationship for inhibition of superoxide production in the cell-free assay by increasing concentrations of the synthetic gp91-*phox* carboxyl-terminal peptide RGVHFIFNKENF. Inhibition of superoxide production in the cell-free assay by various concentrations of RGVHFIFNKENF was measured as described under Materials and Methods and expressed as percent inhibition of superoxide production of control samples in the absence of peptide. Percent inhibition was calculated from $V_{max(peptide)}$ (maximal rate of superoxide production by peptide-containing samples) and $V_{max(control)}$ using eq 1. Shown are means and standard errors of data from three identical experiments (closed circles). The graphed line represents nonlinear regression fit of the modified Hill equation (eq 2) to experimental data (R = 0.96). The calculated value of IC_{50} for RGVHFIFNKENF was 28 ± 1 μM.

Table I: Dependence of Synthetic gp91-*phox* Carboxyl-Terminal Peptide Inhibition of Superoxide Production on the Primary Amino Acid Sequence[a]

peptide sequence	gp91-*phox* amino acid residues	IC_{50} (μM)
ESGPRGVHFIFNKENF (4)	555–570	85 ● 5
RGVHFIFNKENF (3)	559–570	28 ± 1
PRGVHFIFNK (3)	558–567	188 ± 8
RGVHFIF (3)	559–565	38 ± 2
RGVHFI (3)	559–564	>400
RGVHF (5)	559–563	>400
GVHFIF (5)	560–565	300–400

[a] Solubilized membrane (0.5 μg of protein/well) and a range of concentrations of synthetic gp91-*phox* carboxyl-terminal peptides were added to microtiter plate wells and subsequently activated by addition of cell-free assay reaction mixture containing cytosol (16 μg of protein/well) and arachidonate (final concentration 30 μM). Inhibition of superoxide production by peptide was calculated from the V_{max} of peptide-containing samples and controls without peptide as described under Materials and Methods. IC_{50} (peptide concentration that inhibits 50% of control sample superoxide production) was determined in two ways. For RGVHFIF-containing peptides, IC_{50} was determined by nonlinear regression fit of the modified Hill equation to experimental data (see Materials and Methods and Figure 1). Values of IC_{50} are expressed as calculated values ± SEM. For remaining synthetic peptides in this table, a concentration range containing IC_{50} was determined. Numbers in parentheses indicate the number of replicate experiments.

al., 1990; Kleinberg et al., 1990). A representative example of dose-dependent inhibition of superoxide production in the cell-free assay by synthetic gp91-*phox* carboxyl-terminal peptides is shown in Figure 1. Increasing concentrations of RGVHFIFNKENF added to the cell-free assay led to progressive inhibition of superoxide production (Figure 1, closed circles). The ability of synthetic gp91-*phox* carboxyl-terminal peptides to inhibit superoxide production was quantitated by measurement of the concentration of peptide that inhibited 50% of NADPH oxidase activity in the absence of peptide (IC_{50}). IC_{50}'s for the series of truncated gp91-*phox* carboxyl-terminal peptides are shown in Table I. RGVHFIFNKENF and RGVHFIF had similar inhibitory activities to the previously reported gp91-*phox* carboxyl-terminal peptide SNSESGPRGVHFIFNKENF (IC_{50} of 32 ± 2 μM) (Rotrosen et al., 1990) while ESGPRGVHFIFNKENF and PRGVHFIFNK were moderately less inhibitory. In contrast, peptides lacking the complete RGVHFIF sequence had IC_{50}'s

Table II: Dependence of Synthetic gp91-*phox* Carboxyl-Terminal Peptide Inhibition of Superoxide Production on Properties of Individual RGVHFIF Amino Acids[a]

peptide sequence	IC_{50} (μM)	peptide sequence	IC_{50} (μM)
RGVHFIF (3)	38 ± 2	RGVAFIF (3)	53 ± 1
AGVHFIF (3)	300–400	RGVHAIF (5)	>400
RAVHFIF (4)	115 ± 3	RGVHFAF (3)	300–400
RGAHFIF (5)	300–400	RGVHFIA (5)	>400

[a] Solubilized membrane (0.5 μg of protein/well) and a range of concentrations of alanine-substituted RGVHFIF peptides were added to microtiter plate wells and subsequently activated by addition of cell-free assay reaction mixture containing cytosol (16 μg of protein/well) and arachidonate (final concentration 30 μM). Inhibition of superoxide production by alanine-substituted peptides was calculated from the V_{max} of peptide-containing samples and controls containing buffer as described under Materials and Methods. For the inhibitory peptides RGVHFIF, RAVHFIF, and RGVAFIF, IC_{50} was determined by nonlinear regression fit of the modified Hill equation to experimental data (see Materials and Methods and Figure 1). Values of IC_{50} are expressed as calculated values ± SEM. For remaining peptides in this table, a concentration range containing IC_{50} was determined. Numbers in parentheses indicate the number of replicate experiments.

greater than 300 μM and were >8-fold less inhibitory than SNSEGPRGVHFIFNKENF, RGVHFIFNKENF, or RGVHFIF (Table I). GVHFIF and RGVHFI, which differ from RGVHFIF by a single amino acid, were poor inhibitors of superoxide production in the cell-free assay. This suggested that RGVHFIF was the core gp91-*phox* carboxyl-terminal sequence responsible for inhibiting NADPH oxidase activity.

Having established that RGVHFIF was the shortest gp91-*phox* carboxyl-terminal peptide fully capable of inhibiting superoxide production in the cell-free assay, we examined the relative contributions of each RGVHFIF amino acid to overall inhibitory activity. We speculated that the properties of one or more RGVHFIF amino acids were likely responsible for the ability of RGVHFIF to inhibit superoxide production. We synthesized a series of peptides differing from RGVHFIF by substitution of a single alanine for each amino acid of RGVHFIF in order to assess systematically the contribution of individual amino acid side chains to the inhibitory activity of RGVHFIF. Because if its small size, lack of charge, neutral polarity, and preservation of peptide structure, alanine substitution for native amino acids has been used to estimate the effect of the absence of the original amino acid side chain on peptide function (Glass et al., 1989, O'Donnell et al., 1991). The ability of alanine-substituted RGVHFIF peptides to inhibit superoxide was compared to RGVHFIF in the cell-free assay (Table II). Substitution of alanine for the RGVHFIF arginine (gp91-*phox* residue 559) or for the hydrophobic amino acids valine, isoleucine, or either phenylalanine (residues 561, 563–565) was accompanied by marked loss of peptide inhibitory activity compared to RGVHFIF (Table II). These results indicated that the RGVHFIF arginine, valine, isoleucine, and both phenylalanines were critical for expression of inhibitory activity by RGVHFIF. In contrast, RAVHFIF and RGVAFIF, where alanine was substituted for the RGVHFIF glycine or histidine (gp91-*phox* residues 560 and 562), inhibited superoxide production in the cell-free assay though RAVHFIF was less potent than RGVHFIF. These findings suggested that glycine and histidine did not contribute significantly to the inhibitory activity of RGVHFIF.

Discussion

The mechanism of activation and assembly of the NADPH oxidase from its membrane and cytosol components is poorly understood despite cloning and sequencing of cDNA for several oxidase components. We and others have implicated cyto-

chrome b_{558} as a potentially important regulator of activation and formation of the NADPH oxidase (Rotrosen et al., 1990; Heyworth et al., 1991; Kleinberg et al., 1990). Interactions of cytochrome b_{558} with other oxidase components may be mediated by one or more critical gp91-*phox* peptide domains including the gp91-*phox* carboxyl terminus (Rotrosen et al., 1990). Addition of micromolar concentrations of synthetic gp91-*phox* carboxyl-terminal peptides to a cell-free assay of neutrophil NADPH oxidase activity specifically inhibited superoxide production (Figure 1). Other synthetic peptides, including some with sequences corresponding to the amino-terminus of gp91-*phox* and the carboxyl termini of other membrane proteins, possessed no inhibitory activity (Rotrosen et al., 1990).

Synthetic gp91-*phox* carboxyl-terminal peptides inhibit superoxide generation by blocking an early stage of assembly of the NADPH oxidase from cytosol and membrane components (Kleinberg et al., 1990). To identify the core peptide sequence responsible for inhibition of NADPH oxidase activity, we synthesized a series of truncated peptides spanning gp91-*phox* residues 552–570 and examined their abilities to inhibit superoxide generation in a cell-free assay of neutrophil NADPH oxidase activity. Only gp91-*phox* carboxyl-terminal peptides containing the complete RGVHFIF sequence (gp91-*phox* amino acids 559–565) inhibited superoxide generation at concentrations <300 μM (Table I). Results of alanine-substituted peptide experiments suggest that inhibition of NADPH oxidase activity by RGVHFIF-containing peptides is highly specific. RGAHFIF differs structurally from RGVHFIF by two methyl groups, yet RGAHFIF is a poor inhibitor of superoxide production. Our data suggest that arginine, valine, isoleucine, and both phenylalanines (gp91-*phox* amino acids 559, 561, 563–565) contribute significantly to RGVHFIF inhibitory activity whereas RGVHFIF glycine and histidine (gp91-*phox* amino acids 560 and 562) can be substituted with alanine with little loss of inhibitory activity (Table II).

Flanking sequences to RGVHFIF in the gp91-*phox* carboxyl terminus likely influence overall inhibitory activity. Despite the presence of the core RGVHFIF sequence, PRGVHFIFNK was less inhibitory in the cell-free assay than the related peptides RGVHFIFNKENF and RGVHFIF (Table I). This suggested that the amino-terminal proline and/or carboxyl-terminal Asp-Lys of PRGVHFIFNK decreased the inhibitory activity compared to RGVHFIF. Likewise, ESGPRGVHFIFNKENF is less inhibitory than SNSESGPRGVHFIFNKENF and RGVHFIFNKENF, suggesting that one or more amino acids in Glu-Ser-Gly-Pro (gp91-*phox* amino acids 555–558) may decrease the inhibitory activity of ESGPRGVHFIFNKENF compared to RGVHFIFNKENF.

Cytochrome b_{558} may serve as an anchor for assembly of the NADPH oxidase. The carboxyl terminus of gp91-*phox*, located on the cytoplasmic face of the membrane, may bind other oxidase components after neutrophil stimulation. Some characteristics of a putative binding site for the RGVHFIF region of the gp91-*phox* carboxyl terminus can be inferred from results of alanine-substituted peptide experiments. The requirement of the RGVHFIF valine, isoleucine, and both phenylalanines for inhibitory activity in the cell-free assay suggests that binding of the native gp91-*phox* carboxy terminus to another oxidase component is mediated in part by hydrophobic interactions within the binding site. Similarly, the importance of arginine to RGVHFIF inhibitory activity suggests that there is a critical ionic interaction between the native gp91-*phox* carboxyl-terminus arginine 559 and a polar amino acid within the binding pocket of the oxidase component that binds to gp91-*phox*.

The affinities of RGVHFIF-containing peptides for the binding site of the native gp91-*phox* carboxyl terminus on another phagocyte cannot be determined explicitly from measured values of IC_{50}. The magnitude of IC_{50} depends not only on the affinity of the synthetic peptide for its binding site but also on the concentrations of cytochrome b_{558} and other oxidase components as well as the affinity of the native gp91-*phox* carboxyl terminus for its binding site. Values of IC_{50} can be considered as lower limits of the affinity of RGVHFIF-containing peptides for the gp91-*phox* carboxyl-terminal binding site. Actual affinities may be considerably greater especially if the affinity of the native gp91-*phox* carboxyl terminus for its binding site is high.

In conclusion, we have identified an RGVHFIF-containing gp91-*phox* domain that likely mediates cytochrome b_{558} participation in the assembly of the NADPH oxidase after phagocyte stimulation. The activity of this RGVHFIF-containing domain appears to be mediated by a critical, positively charged arginine at the RGVHFIF amino terminus and the hydrophobicity of valine, isoleucine, and phenylalanines at the carboxyl terminus. Further characterization of properties of gp91-*phox* carboxyl-terminal peptides that inhibit superoxide production may facilitate understanding the mechanism of formation of the NADPH oxidase.

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Registry No. Arg, 74-79-3; Val, 72-18-4; Ile, 73-32-5; Phe, 63-91-2; NADPH oxidase, 9032-22-8; cytochrome b_{558}, 9064-78-2.

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One-Step Purification of *Actinoplanes missouriensis* D-Xylose Isomerase by High-Performance Immobilized Copper-Affinity Chromatography: Functional Analysis of Surface Histidine Residues by Site-Directed Mutagenesis†

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ABSTRACT: D-Xylose isomerase (XI) is a heat-stable homotetrameric enzyme used in industry for the production of high-fructose corn syrups by isomerization of D-glucose into D-fructose. To carry out biochemical and structural studies of this enzyme and of its engineered variants, a rapid and convenient method of purification of recombinant *Actinoplanes missouriensis* XI produced in *Escherichia coli* has been developed. The availability of surface-accessible histidine residues allows adsorption of XI to immobilized metal-affinity chromatography (IMAC) columns. Knowledge of the physicochemical properties of this enzyme is shown to further warrant rational modifications in the composition of the chromatographic solvents so as to achieve high selectivity in both its interaction with and its elution from a copper-loaded Chelating Sepharose Fast Flow column, an agarose-based matrix derivatized with iminodiacetic acid (IDA) groups. Purification of XI to homogeneity can thus be accomplished in a single chromatographic step starting from crude cell lysates. IDA–Cu(II)–IMAC proves convenient, fast, and reproducible. Moreover, this method is gentle to and hence suitable for mutant enzymes with decreased stability. Its disadvantage is that XI is purified in an inactive form due to inhibition by scavenged Cu^{2+}. This handicap is however easily overcome by means of a polishing step by chromatography on Mono-Q in the presence of the chelator, EDTA. Site-directed mutants have been constructed to assess the role of surface amino acid residues in the IMA recognition event. Substitution of lysine for histidine 41 results in a mutant with near wild-type properties. Yet, this mutation is shown to completely abolish adsorption to IDA–Cu(II). This finding is analyzed in relation to the structural surface properties of the XI enzyme to provide direct evidence for the implication of histidine 41 as the predominant protein ligand to IDA–Cu(II) in IMAC.

Due to rapid advances in genetic engineering, numerous proteins can now be produced efficiently in foreign host cells. Recombinant DNA technology permits replacement of any amino acid in a protein. This strategy is used in protein engineering in an effort to understand how protein structure relates to function. Such knowledge is essential to eventually design a protein with improved properties, and it requires the detailed chemical–physical and structural characterization of the wild type and of several genetically engineered variants.

The technology for purifying recombinant proteins has however lagged behind the technology for cloning the genes expressing them. When multiple variants need to be purified concurrently, it is convenient that the purification protocol be (i) simple, i.e., involves as few steps as possible; (ii) rapid, in order to avoid having to compare an "aged" reference sample with a freshly obtained mutant protein; and (iii) applicable to a large number of the protein variants being investigated.

This laboratory has been involved in the past five years in a protein engineering project aimed at improving the properties of D-xylose isomerase (XI;[1] EC 5.3.1.5) from *Actinoplanes missouriensis*. Microbial xylose isomerases are large, tet-

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[1] Abbreviations: XI, D-xylose isomerase; HPLC, high-performance liquid chromatography; IMA, immobilized metal affinity; IMAC, immobilized metal-affinity chromatography; IDA, iminodiacetic acid; IDA–Me(II) chelate complex, where Me(II) represents any divalent metal ion; IDA–Cu(II)–IMAC, IMAC with IDA–Cu(II) as affinity ligand; ASA, accessible surface area; SDH, D-sorbitol dehydrogenase; MOPS, 4-morpholinepropanesulfonic acid; EPPS, 4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid; MES, 4-morphineethanesulfonic acid; TEA, triethanolamine; Tris, Tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; kDa, kilodaltons; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

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